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UNIVERSITY OF ALBERTA

In vivo and *in vitro* characterization of *Saccharomyces cerevisiae* tRNA$^{\mathrm{Trp}}$ suppressor constructs

BY
Audrey L. Atkin ©

A THESIS SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF GENETICS

EDMONTON, ALBERTA
Spring, 1992

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FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the faculty of graduate studies and research for acceptance, a thesis entitled *IN VIVO* AND *IN VITRO* CHARACTERIZATION OF *SACCHAROMYCES CEREVISIAE* tRNA^{TRP} SUPPRESSOR CONSTRUCTS, submitted by AUDREY L. ATKIN in partial fulfilment of the requirements for the degree of DOCTOR OF PHILOSOPHY.

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This thesis is dedicated to my parents for all their support and encouragement, and to Jim for his patience.

ABSTRACT

Suppressor alleles of the *Saccharomyces cerevisiae* tRNA^{Trp} genes have not been isolated by conventional genetic techniques, consequently *in vitro* mutagenesis was used to construct potential suppressor alleles. Amber suppressor allele of this gene encode efficient and specific suppressors of UAG codons when present on single copy plasmids. The opal suppressor is also specific, however it is a very weak suppressor. A second change in the intron of this suppressor opal allele creates a more efficient suppressor. Analysis of RNA isolated from yeast cells transformed with the opal suppressor alleles indicated that the difference in suppressor efficiency may be due to less effective processing of the intron from precursors of the gene containing only the requisite anticodon change. It was hypothesized that this may reflect an altered secondary structure of the precursor in which base-pairing at the 3' splice junction inhibits splicing. This was tested by engineering additional changes into the anticodon and the intron of this tRNA^{Trp} gene. Mutations in the anticodon of the tRNA^{Trp} gene, which should allow base-pairing at the 3' splice site, were shown to affect processing of pre-tRNA^{Trp} at the intron removal step. Second site mutations in the intron, designed to inhibit base-pairing at the 3' splice junction, could restore recognition and cleavage by the tRNA splicing endonuclease if another step in the processing pathway has not been disrupted. In an attempt to understand why the isolation of tRNA^{Trp} suppressors, by conventional genetic techniques, has not

been published, the family of $tRNA^{Trp}$ genes from *S. cerevisiae* were characterized. This family has six members, identical in sequence, and distributed on five or six chromosomes. Replacement of a copy of a $tRNA^{Trp}$ gene demonstrated that a maximum of five $tRNA^{Trp}$ genes is sufficient for viability of haploid yeast cells. These results, and attempts to replace a copy of the $tRNA^{Trp}$ gene with an engineered amber suppressor allele, suggest that suppressor alleles of this gene may not have been obtained by conventional genetic techniques because the $tRNA^{Trp}$ genes are not expressed as efficiently in their native chromosomal loci as they are on plasmids.

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TABLE OF CONTENTS

Chapter	Pages
I. Introduction	1
Literature cited	47
II. Construction of an opal suppressor by oligonucleotide directed mutagenesis of a *Saccharomyces cerevisiae* tRNA^{Trp} gene	67
Literature cited	99
III. Processing of precursor transcripts from a *Saccharomyces cerevisiae* tRNA^{Trp} gene may be affected by mutations in the anticodon or in the intron	104
Literature cited	132
IV. Characterization of the tRNA^{Trp} genes of *Saccharomyces cerevisiae*	136
Literature cited	162
V. SUMMARY AND CONCLUSIONS	165
Literature cited	179

LIST OF TABLES

Table	Description	Page
I-1	The tRNAs which should require a single change in the anticodon to produce amber, ochre or opal suppressors	7
II-1	Suppressor phenotype of the yeast strains when transformed with wild type and engineered tRNA^{Trp} genes	96
II-2	Free energies for the secondary structure of the anticodon stem, loop and intron of the precursor tRNA^{Trp} transcripts for each of the two possible conformations	98
III-1	Suppressor phenotype of yeast strains when transformed with wild type and engineered tRNA^{Trp} genes	130
IV-1	Yeast strains used in these studies	153

LIST OF FIGURES

Figure	**Description**	**Page**
I-1	Pre-tRNA splicing mechanism in *Saccharomyces cerevisiae*	30
II-1	Sequence of the wild type and engineered tRNA^{Trp} genes	86
II-2	Cloverleaf structures of wild type and engineered pre-tRNA^{Trp}	88
II-3	Northern hybridization analysis of the tRNA^{Trp} transcripts isolated from the transformants	90
II-4	The wild type pre-tRNA^{Trp} may have two alternative secondary structures	92
II-5	The alternative secondary structures for the anticodon stem, loop, and the intron of precursors transcribed from op1	94
III-1	Primary sequence and possible secondary structures of the *Saccharomyces cerevisiae* tRNA^{Trp} precursor	124

III-2 The pattern of cleavage products produced by tRNA slicing endonuclease digestion of gel purified precursors transcribed from the wild type and engineered tRNA^{Trp} genes and the *SUP53* gene 126

III-3 Northern hybridization analysis of the tRNA^{Trp} transcripts isolated from transformants 128

IV-1 Southern blot analysis of genomic DNA from *Saccharomyces cerevisiae* strain RJ293-13C and JG369-3B 154

IV-2 Southern blot hybridization of yeast chromosomes separated by pulse gel electrophoresis 156

IV-3 Sequence of a cloned tRNA^{Trp} gene 158

IV-4 Single-step gene replacement of a chromosomal copy of a tRNA^{Trp} gene with the *URA3* gene 160

SYMBOLS, NOMENCLATURE OR ABBREVIATIONS

AARs	aminoacyl-tRNA synthetases
bp	Base pair
Ci	Curie
cpm	Counts per minute
D	Dihydrouridine
50XDenhardt's	1% (w/v) ficoll, 1% (w/v) polyvinyl pyrrolidone, 1% (w/v) bovine serum albumin (pentax, fraction V)
EDTA	Ethylenediaminetetraacetic acid
fmet	N-formyl methionine
ΔG_{37}	Free energy change at 37°C
i^6A	Isopentenyladenosine
IVS	Intervening sequence
kb	Kilobase
kDA	Kilodalton
K_m	Michaelis-Menten constant
mCm^5S^2U	5-methoxycarbonyl methyl-2-thiouridine
mRNA	Messenger ribonucleic acid
N	Normal
nm^5U	5-amino methyluridine
NTP	Nucleoside triphosphate
pCp	Cytosine 5'-monophosphate-3'-monophosphate
PCR	Polymerase chain reaction
pre-tRNA	Transfer ribonucleic acid precursor transcript
RNase P	Ribonuclease P

SDS	Sodium dodecyl sulfate (lauryl sulfate)
20XSSC	3M NaCl, 0.3M Na citrate (pH 7.0)
TFIIIB	Transcription factor III B
TFIIIC	Transcription factor III C
Tris	Tris (hydroxymethyl) amino-methane
tRNA	Transfer ribonucleic acid
yW	Wybutosine

CHAPTER I: INTRODUCTION

Informational suppression in *Saccharomyces cerevisiae* has been studied for approximately three decades. To date nonsense suppressor alleles of only $tRNA^{Tyr}$, $tRNA^{Ser}$, $tRNA^{Leu}$ and $tRNA^{Gln}$ genes have been found by conventional genetic techniques (reviewed in Sherman, 1982; and Edelman and Culbertson, 1991). Suppressor alleles of the six other tRNA genes which would require a single base change in the anticodon to create a tRNA capable of recognizing one of the nonsense codons have not been isolated by traditional techniques. The tryptophan tRNA is one of these. In this thesis, site-directed mutagenesis was employed to construct amber and opal suppressor alleles of a yeast $tRNA^{Trp}$ gene. In addition, the family of yeast $tRNA^{Trp}$ genes were cloned and partially characterized. The $tRNA^{Trp}$ genes are one of ten families of tRNA genes which encode introns (Ogden et al., 1984 and Stucka and Feldmann, 1988). A comparison between the members of the intron-containing tRNA gene families suggests that the family of $tRNA^{Trp}$ genes is unique because of the lack of polymorphism within the intronic sequences of the $tRNA^{Trp}$ genes.

The introduction to this thesis attempts to provide a general overview of nonsense suppression. The steps involved in the production of functional suppressors are reviewed. These steps include transcription of the tRNA gene, 5' and 3' end-processing of the precursor transcript, intron-removal when intervening sequences are present, and aminoacylation of the mature tRNA. In addition, nucleotide modifications are discussed because, although they do not play an absolute role in suppression, they can modulate the efficency of suppression. Finally, a compilation of the tRNA genes which encode introns has been included.

The genetic code and nonsense suppression:

In the genetic code, there are 64 three base codons. Sixty-one of these correspond to a specific one of the 20 amino acids. The three others (UGA, UAA and UAG) usually act as codons specifying termination of polypeptide synthesis and are generally found at the end of a translational unit. In certain codons, mutation can lead to the presence of nonsense codons in a mRNA at a position which would normally code for an amino acid. This results in premature termination of protein synthesis. These mutations can be suppressed by second site mutations which prevent the premature termination of protein synthesis. With a few exceptions, nonsense suppressors are tRNA genes wherein a mutation has occurred, usually in the anticodon, which alters the codon specificity of the tRNA.

It was first hypothesized that nonsense suppression is mediated by an altered tRNA by Benzer and Champe (1962). They showed that some mutations in the rII region from phage T4 could be suppressed by second site mutations on the host chromosome. In addition, these suppressors could suppress a subset of mutations in the structural cistron of alkaline phosphatase in *Escherichia coli*. Garen and Siddiqi (1962) demonstrated that the alkaline phosphatase mutants produce no detectable phosphatase protein in the absence of the suppressor, as would be expected if nonsense mutations were involved. In the presence of the suppressor between three and 100% enzyme activity was detected, depending upon the suppressible phosphatase mutant. Based on the results of these companion publications, Benzer and Champe proposed that nonsense suppression is mediated by tRNA.

Shortly after the publication of these papers, the discovery of an analogous class of mutants in *S. cerevisiae* was published. Hawthorne and Mortimer (1963) described two nonallelic suppressors which are capable of suppressing mutations at fourteen loci in this yeast. These suppressors are effective in restoring the wild type character for only a fraction of the mutant alleles at a given locus.

Further evidence for the hypothesis that an altered tRNA mediates suppression in *E. coli* was provided by two independent research groups. Capecchi and Gussin (1965) isolated RNA from a strain of an RNA bacteriophage (R17) which has an amber (UAG) mutation in the coat protein cistron. This RNA was capable of directing the synthesis of functional coat protein in a cell free protein-synthesis system derived from a permissive host. No functional coat protein is produced in a system derived from a nonpermissive host. In a mixed system, consisting of serine-accepting tRNA from a permissive host added to the extract from a nonpermissive host, functional coat protein is produced. A similar system, using the coat protein gene from phage f2, was used to demonstrate the insertion of serine into the protein at the site of a nonsense mutation, which is mediated by tRNA isolated from a permissive host (Engelhardt et al., 1965). Neither of these studies could distinguish between mutated tRNAs and mutated tRNA-modifying enzymes as the suppressors. In a paper documenting the isolation of additional yeast suppressors, an elegant argument was made for the hypothesis that yeast suppressors are altered tRNAs (Magni et al., 1966).

Direct evidence that altered tRNAs mediate suppression came from the sequencing of the tyrosyl tRNA specified by the *Su3⁻* gene of *E. coli* (Goodman et al., 1968). The sequence of this tRNA differed from the sequence of the tRNA^{Tyr} encoded by the wild type gene (*Su3⁺*) by a single base in the anticodon. Nonsense suppressor tRNAs, able to interact with each of the termination codons have been identified from *E. coli*. Amber suppressor tRNAs are capable of reading the UAG stop codon, while ochre and opal suppressor tRNAs are capable of reading the UAA and UGA stop codons, respectively. Interestingly, the ochre suppressor tRNAs are capable of wobble reading and may suppress amber as well as ochre codons, and all opal suppressors are also capable of reading the codon for tryptophan in *E. coli* . Suppressor alleles of the *E. coli* tRNA genes encoding $tRNA^{Gln}$, $tRNA^{Leu}$, $tRNA^{Ser}$, $tRNA^{Trp}$, $tRNA^{Tyr}$, $tRNA^{Lys}$, $tRNA^{Gly}$, and $tRNA^{Glu}$ have been isolated by conventional genetic techniques. Most of these suppressor alleles encode tRNA with an altered anticodon (reviewed in Eggertsson and Söll, 1988). The only known example in *E. coli* of tRNA-mediated suppression of premature termination being caused by a mutation at a site outside the anticodon triplet is an opal suppressor allele of the gene for $tRNA^{Trp}$ (Hirsh, 1971). Recent work has focused on the construction of a complete set of amber suppressor tRNAs, one for each of the 20 naturally occurring amino acids. Amber suppressor genes for $tRNA^{Phe}$ and $tRNA^{Cys}$ were the first of these to be constructed by *in vitro* techniques (Normanly et al., 1986a).

The first demonstration that tRNA also mediates informational suppression in yeast was not done until 1975, presumably because of the need to develop an efficient *in vitro* protein synthesis system (Capecchi et al., 1975). Suppression was assayed by translation of a mRNA for a coat protein from bacteriophage Qβ, with an amber nonsense mutation. An *in vitro* protein synthesis system, derived from mouse tissue culture cells supplemented with tRNA isolated from either a nonsuppressing strain or one of two suppressor strains known to translate through nonsense mutations *in vivo*, was utilized. Mature-sized Qβ coat protein was also produced in this protein-synthesizing system supplemented with *E. coli* suppressor tRNA, indicating that the biochemical mechanism for nonsense suppression is very similar in yeast and *E. coli.*

Soon thereafter, suppressor alleles from *S. cerevisiae* were also shown to encode tRNA with an altered anticodon. Goodman et al. (1977) sequenced a wild type and a suppressor allele of a $tRNA^{Tyr}$ gene from *S. cerevisiae*. The sequence of the ochre suppressor allele differs from the wild type gene by virtue of a G·C to T·A transversion in the base pair that codes for the wobble position base of the $tRNA^{Tyr}$ anticodon. Extensive screens have been done to isolate nonsense suppressors in yeast. To date, eight tyrosine-inserting suppressors have been isolated which correspond to the eight $tRNA^{Tyr}$ genes. Five serine-inserting suppressors have been identified, and there are eleven leucine-inserting suppressors (reviewed in Sherman, 1982). In addition, an amber suppressor allele (*SUP70*) of the yeast glutamine $tRNA_{CAG}$ has been identified (Edelman and Culbertson, 1991). Ochre suppressor alleles resulting from second site changes in *SUP70* have also been isolated (Edelman and Culbertson, 1991). In contrast to the situation in *E. coli*, ochre suppressors in yeast are not able to read the amber nonsense codon as well as the ochre codon. Also, suppressor alleles of the six other tRNA genes, predicted to be able to result from a single base change in the anticodon to create a tRNA capable of base pairing with one of the nonsense codons (Table I-1), have not been isolated by conventional techniques. However, wild type $tRNA^{Gln}$ can suppress ochre and amber nonsense mutations, and wild type $tRNA^{Trp}$ can suppress opal nonsense mutations when these genes are present on

TABLE I-1:

The tRNAs which should require a single change in the anticodon to produce amber, ochre or opal suppressors.

tRNA	Codon	AMBER	OCHRE	OPAL
tRNA^{Arg}	CGA			+
	AGA			+
tRNA^{Cys}	UGC			+
	UGU			+
tRNA^{Glu}	GAA		+	
	GAG	+		
tRNA^{Gln}	CAA		+	
	CAG	+*		
tRNA^{Gly}	GGA			+
tRNA^{Leu}	UUA		+*	+*
	UUG	+*		
tRNA^{Lys}	AAA		+	
	AAG	+		
tRNA^{Ser}	UCA		+*	+*
	UCG	+*		
tRNA^{Trp}	UGG	+		+
tRNA^{Tyr}	UAU	+*	+*	
	UAC	+*	+*	

*Suppressor alleles have been isolated by conventional genetic techniques in *Saccharomyces cerevisiae.*

high copy number plasmids (Pure et al., 1985; Weiss and Friedberg, 1986; Atkin et al., 1990; and Kim et al., 1990).

In vitro techniques have also been used to construct suppressor alleles of various yeast tRNA genes. The first example was the synthesis and expression of amber and ochre suppressor alleles of a $tRNA^{Phe}$ gene (Masson et al., 1987). Since that time it has been reported that amber and opal suppressor alleles of a $tRNA^{Trp}$ gene can be constructed by site-directed mutagenesis (Kim and Johnson, 1988; Atkin et al., 1990; and Kim et al., 1990).

Nonsense suppression has been studied in a variety of other eukaryotes. Ochre, opal and amber suppressors have been selected by conventional genetic techniques in *Schizosaccharomyces pombe* (Krupp et al., 1985). Interestingly, an opal suppressor of *S. pombe*, *Sup3-e*, is a functional suppressor in *S. cerevisiae*, however the *SUPRL1*, $tRNA^{Tyr}$ and $tRNA^{Leu}$ genes of *S. cerevisiae* are not transcribed in *S. pombe* (Hottinger et al., 1982; and Krupp, et al., 1985).

To date *Caenorhabditis elegans* is the only multicellular organism which has allowed the recovery of nonsense suppressors through classical genetics. Eight of the 12 $tRNA^{Trp}$ genes have been converted to amber suppressor alleles. These amber alleles have been used to show that these genes are expressed differentially in a cell-type or developmental stage-specific manner (Kondo et al, 1990).

Informational suppressor tRNAs have not been isolated by classical genetics in *Drosophila*. However, the availability of P-element mediated transformation has made the study of nonsense suppression in *Drosophila* feasible. Transformations have been done with *Sup3-e* gene of *S. pombe* (Molnar et al., 1988). *Sup3-e* was chosen for these experiments because it does suppress a known *Drosophila* nonsense mutant *in vitro*, as ascertained by an immunological assay (Kubli et al., 1982). *Sup3-e* does not appear to suppress the same mutation *in vivo*. The reason for this is not yet understood (Molnar et al., 1988). Site-directed *in vitro* mutagenesis has been used to create suppressor alleles of *Drosophila melanogaster* $tRNA^{Tyr}$, $tRNA^{Leu}$ and $tRNA^{Ser}$ genes (Doering et al., 1988; Garza et al., 1990; and Pappu et al., 1990). The putative suppressor alleles of the $tRNA^{Leu}$ and $tRNA^{Ser}$ genes were tested in *S. cerevisiae* before transformation into *Drosophila*. The suppressor alleles of these genes functioned in yeast with strict codon specificity (Garza et al., 1990; and Pappu et al., 1990). A very low level of suppression was observed in flies transformed with an amber $tRNA^{Tyr}$ suppressor (Doering et al., 1988). No suppression was observed in flies transformed with the suppressor alleles of the $tRNA^{Leu}$ or $tRNA^{Ser}$ genes (Garza et al., 1990; and Pappu, 1990). Both the $tRNA^{Tyr}$ and $tRNA^{Leu}$ suppressor transformants had poor viability (Doering et al., 1988; and Garza et al., 1990). It is likely that suppressors have not been selected in flies by conventional genetics because of the toxicity of these suppressors, presumably due to interference with normal chain termination.

Classical informational suppressors have not been found in mammalian cells, but so called natural suppressor tRNAs can be isolated from wild type cells (O'Neill et al., 1985; Kuchino et al., 1987; Valle et al., 1987; and Sedivy et al., 1987). These tRNAs were originally discovered when examining translation of certain viral mRNAs. Read through of termination codons in viral mRNAs appears to be a common mechanism of regulating gene expression in plant and animal RNA viruses (reviewed in Kozak, 1986). It has been proposed that these tRNAs have specialized functions in higher eukaryotes such as the insertion of phosphoserine directly into protein in response to specific UGA codons (O'Neill et al., 1985).

Although information about nonsense suppression is accumulating in a variety of eukaryotic organisms, *S. cerevisiae* is still the most well defined system for the study of nonsense suppression. Thus it is the organism of choice for detailed studies of tRNA at the molecular level.

Modifiers of nonsense suppressors:

The expression of suppressors in *S. cerevisiae* can be affected by the genetic backgrounds in which the suppressors exist. Two classes of mutants have been found. The first class, allosuppressors, enhance the expression of suppressors, while the second class, antisuppressors, decrease suppressor efficiency.

The ψ determinant and certain chromosomal mutations can act as allosuppressors. The ψ extrachromosomal determinant can enhance the expression of ochre, but not amber or opal suppressors. It was originally identified by its ability to enhance the expression of the ochre suppressor encoded by *SUP16* (Cox, 1965). *SUP16* is a mutant allele of a gene in a family which consists of three genes for $tRNA^{Ser}$ with the UCA anticodon. ψ similarily influences the expression of suppressor alleles, *SUP17* and *SUP19,* of the other two genes (Ono et al., 1979). All $tRNA^{Tyr}$ ochre suppressors are very efficient, and are lethal or sublethal in a ψ^+ background (Cox, 1971). The recessive chromosomal mutations, *sal1 - sal5*, increase the efficiency of at least one amber suppressor and all UAA suppressors (Cox, 1977). In addition, these allosuppressors, in combination with a tyrosine-inserting, ochre-specific suppressor, will suppress amber alleles fairly efficiently. These allosuppressors also increase the expression of the tyrosine-inserting suppressors to a level that causes lethality or reduced viability. Recently, an additional five mutations resulting in UAG suppression which is strictly dependent on UAA suppression by a resident *SUP4-o* gene have been genetically characterized (Gelugne and Bell, 1988). In addition, wild type $tRNA^{Trp}$ can suppress opal alleles when present on a multicopy plasmid in two different genetic backgrounds, but not in a third (Atkin et al., 1990; and Kim et al., 1990). The mechanism which allows the CCA anticodon of the tRNA^{Trp} to interact with the UGA codon is probably analogous to the mechanism which enables ochre suppressors to read amber nonsense codons.

All antisuppressors isolated to date have been in chromosomal genes. Eight distinct antisuppressors, *asu1* - *asu8*, reduce the activity of UAA suppressors *SUP11*, *SUP2* and *SUP16*, but do not appear to affect a UAG suppressor, *SUP11* (McCready and Cox, 1973). The antisuppressors, *mod1* - *mod6* and *moda* reduce the activity of a UAA suppressor, *SUP7* (J. Gorman et al. [unpublished] cited in Laten et al., 1978). The biochemical defect has been characterised for *mod5*. In a *mod5* genetic background the tRNA has only 1.5% of the normal level of isopentenyladenosine (Laten et al., 1978). The *MOD5* gene has been cloned and shown to encode the enzyme responsible for modification of adenosine to isopentenyladenosine at position 37 adjacent to the 3' end of the anticodon (Dihanich et al., 1987) The dominant antisuppressor *SIN2* can affect UAG suppressors which have second site mutations, making these suppressors susceptible to *SIN2* (Hawthorne and Leupold, 1974). Recently, two genes, *LOS1* and *STP1*, have been characterised which are involved in intron removal from pre-tRNA (Hurt et al., 1987; and Wang and Hopper, 1988). *LOS1* and *STP1* mutants reduce the expression of *SUP4* and thus behave as antisuppressors. Since these genes are involved in intron removal from pre-tRNA, they may behave as antisuppressors for all tRNA^{Tyr} and tRNA^{Leu} suppressors, and the amber suppressor alleles of tRNA^{Ser}, since all of these genes have introns.

The tRNA genes of *S. cerevisiae* which encode introns:

There are 10 families of tRNA genes which encode introns. The precursors of 9 of these tRNA isoacceptors accumulate in *rna1-1* mutants (Ogden et al.,1984). The *rna1-1* mutant strain was originally isolated as a temperature sensitive defect in the maturation of mRNA and was subsequently shown to also accumulate tRNA precursors (Hopper et al., 1978). O'Farrell et al. (1978) isolated intron containing precursors for tRNA^{Tyr} which accumulate in an *rna1-1* strain, and showed that these precursors contain an intervening sequence (IVS) identical to that found in the gene for tRNA^{Tyr}. Although this pre-tRNA is functionally inactive, a crude nuclear extract from yeast containing an excision-ligase activity was capable of processing pre-tRNA^{Tyr} into mature tRNA^{Tyr}. Additional intron-containing precursors for tRNA^{Phe}, tRNA$^{Ser}{}_{UCG}$, tRNA$_3{}^{Leu}$ and tRNA^{Trp} were sequenced, and found to be substrates for the yeast tRNA excision-ligase activity (Knapp et al., 1978; and Knapp et al., 1979). Ogden et al. (1984) completed the analysis of precursors that accumulate in *rna1-1* strains. Nine cytoplasmic tRNA isoacceptors have been identified that are derived from intron-containing pre-tRNAs. A tenth tRNA isoacceptor, whose intron-containing precursors were not detected or do not accumulate in *rna1-1* mutants, was recently reported (Stucka and Feldman, 1988).

The gene for tRNA^{Tyr} was the first of the intron containing tRNA genes to be cloned (Goodman et al., 1977). Eight unlinked suppressor loci, *SUP2* to *SUP8* and *SUP11* have been shown to insert tyrosine at nonsense codons in yeast iso-1-cytochrome C (reviewed in Sherman, 1982). A single nucleotide sequence has been observed for tyrosine tRNA in wild type yeast strains, although a ^{125}I-labelled tRNA^{Tyr} hybridizes to eight *Eco*R1 fragments of *S. cerevisiae* DNA. In conjunction with genetic evidence, this suggests that there are eight unlinked genes which code for tRNA^{Tyr} (Olson et al., 1977). Three of the eight unlinked genetic loci, which all can mutate to tyrosine-inserting nonsense suppressors, have been cloned (Goodman et al., 1977). All encode the same mature tRNA sequence, however all three of the DNA sequences differ from that of yeast tRNA^{Tyr} by virtue of a 14 base pair intron:

ATTTAYCACTACGA
TAAATRGTGATGCT

(where Y=pyrimidine, R=purine), located one base 3' to the anticodon. The intron of the tRNA^{Tyr} *SUP6* gene was deleted to test whether or not it is essential for the expression of the gene. The altered tRNA gene encoded an active suppressor, demonstrating that the intervening sequence is not absolutely necessary for the expression of this gene (Wallace et al., 1980). Further tests showed that removal of the IVS converts the *SUP6* gene to a less efficient suppressor. In addition, the IVS is necessary for the modification of uridine in the tRNA anticodon to pseudouridine. It was proposed that the failure to make this modification causes the decrease in suppressor efficiency of the mutant (Johnson and Abelson, 1983).

Two different intron-containing precursor $tRNA^{Phe}$ molecules accumulate in *rna1* mutants. The sequence of these precursors differs only by a base substitution in the intron and an insertion at the same position (Ogden et al., 1984). Ten $tRNA^{Phe}$ genes have been cloned. Three of these have been sequenced (Valenzuela et al., 1978). Two correspond to the precursors which have an 18 base pair intron, and the third matches the sequence of the precursor with a 19 base pair intron. Synthetic ochre and amber tRNA suppressor genes have been constructed, based on the sequence of the yeast phenylalanine $tRNA_{GAA}$ gene encoding a 19 base-pair intron. The amber suppressor allele efficiently suppresses UAG codons, while the ochre suppressor alleles are inefficient suppressors. Both are toxic when present on a high copy number plasmid. Ochre suppressor alleles which lack the intron have the same phenotype as the intron-containing suppressor alleles (Masson et al., 1987), suggesting that the intronic sequence is not necessary for yeast $tRNA^{Phe}$ function.

Three genes for the major leucine-specific tRNA isoacceptor (insert leucine at UUG) have been cloned and sequenced. Two of these genes encode a 32 base pair intron and the third has an intron of 33 base pairs (Kang et al., 1980; Andreadis et al., 1982; and

Standring et al., 1981). The sequences of the genes with the 32 base pair introns are identical (Kang et al., 1980; and Andreadis et al., 1982). The gene containing the 33 base pair intron differs from the two sequenced genes by the insertion of a base and two base substitutions within the IVS. *SUP53*, a leucine-inserting amber suppressor, maps to the same region as one of the two $tRNA^{Leu}$ genes encoding the 32 base pair intron. The products of these genes differ by a single nucleotide substitution in the anticodon, suggesting they are allelic (Andreadis et al., 1982). Additional suppressors which insert leucine have been isolated (Liebman et al., 1976; Liebman et al., 1977; and Ono et al., 1979). Genetic analyses indicate there are at least six loci yielding leucine-inserting suppressors (Sherman, 1982). The intron was removed or mutations were made in the intron of *SUP53* to examine the role of intron sequence and structure on precursor tRNA processing and mature tRNA function. Mutant analysis showed that the totally intact precursor tRNA, rather than any specific sequence or structure of the intron, was necessary for efficient nonsense suppression by *SUP53*. Less efficient suppressor activity correlated with the absence of the 5-methylcytosine modification of the anticodon wobble base (Strobel and Abelson, 1986a). Most alterations in the intron of this gene do not impair processing of precursor tRNAs, unless they perturb the overall structure of the pre-tRNA (Johnson et al., 1980; Raymond and Johnson, 1983; and Szekely et al., 1988). Intron-containing precursor for the leucine-inserting tRNA with a UAG anticodon also accumulates in a *rna1* mutant. To date, the gene for this precursor has not been cloned. These precursors have a 19 base pair intron. Two variants accumulated, which differ at a single base (Ogden et al., 1984).

The genes for two families of serine-inserting tRNAs have introns. There is a single locus for the tRNA which inserts serine at the UCG codon (Etchverry et al., 1979). This gene was initially isolated as a recessive lethal amber suppressor (*SUP-RL1*) (Brandiss et al., 1975). An allelic serine-inserting ochre suppressor (*SUP61*) has also been isolated (Hawthorne and Leupold, 1974). It was shown that this gene is unique by demonstrating that the lethality is due to the loss of wild type function (Etchverry et al., 1982). The gene for this tRNA has been cloned and sequence analysis revealed the presence of a 19 base pair intervening sequence. Interestingly, the gene for *SUQ5*, another minor $tRNA^{Ser}$, does not encode an intron (Olson et al., 1981). Constructs were prepared to assess the role of the intron in the *SUP-RL1* gene on gene expression. Unlike the introns in $tRNA^{Tyr}$ and $tRNA^{Leu}$, which appear to be necessary for base modifications within the anticodon, the removal of this intron had no discernible effect on the expression of this gene (Ho and Abelson, 1988). Recently, a gene for the $tRNA^{Ser}$ which is capable of inserting serine at the AGU and AGC codons was cloned and sequenced. This gene also encodes a 19 base pair intron, however there is no sequence homology to the intron of the *sup-RL1* gene. According to genomic hybridizations, there are three copies of this gene (Stucka and Feldmann, 1988). Precursors transcribed from these genes were not observed to accumulate in *rna1* mutants (Ogden et al., 1984).

The yeast proline tRNAs have been analysed primarily by the isolation of mutations in the tRNA genes that can suppress +1 frameshift mutations in proline codons (Cummins et al., 1980). There are at least six members of this gene family (Cummins et al., 1985). These genes encode introns which vary in sequence and in length from 28 to 31 bases (Ogden et al., 1984; Lee and Knapp, 1985; and Cummins et al., 1985). These proline tRNA genes mutate to suppress +1 frameshift mutations in proline codons via a G to U base substitution mutation at position 39 (Cummins et al., 1985). This mutation alters the 3' splice junction and disrupts the bottom base-pair of the anticodon stem, resulting in a four-base pair anticodon stem and a nine-base anticodon loop which presumably allows the tRNA to read a four-base codon (Winey et al., 1986).

The nucleotide sequence of mature $tRNA^{Trp}$ has been published (Keith et al., 1971) and a gene for $tRNA^{Trp}$ has been cloned (Kang et al., 1980). The sequence of the gene differs from the sequence of the mature tRNA by the presence of a 34 base pair intron. A precursor corresponding to this gene accumulates in *rna1* mutants (Ogden et al., 1984). Southern analysis has been used to determine the copy number of $tRNA^{Trp}$ genes to be six. These genes are distributed on five to six chromosomes (Yesland et al., 1991, and this thesis, Chapter IV). PCR technology has been used to clone and sequence these genes. The sequence of the exons and the intron are identical to the sequence of the gene originally cloned (this thesis, Chapter IV). This tRNA has an anticodon capable of mutating to recognize nonsense codons via a single base substitution. Single changes in the CCA anticodon of $tRNA^{Trp}$ should allow precise base pairing with the amber (UAG) and opal (UGA) codons, and a double change should result in a tRNA which can interact with the ochre (UAA) codon (Kim and Johnson, 1988; and this thesis). *In vitro* mutagenesis has been used to create functional amber and opal suppressor alleles of the $tRNA^{Trp}$ gene (Kim and Johnson, 1988; Atkin et al, 1990; and Kim et al., 1990). Attempts to construct a functional ochre suppressor have not yet been successful (this thesis, Chapter III).

Intron-containing precursors of $tRNA_2^{Lys}$ and $tRNA^{Ile}$ also accumulate in *rna1* mutants (Ogden et al., 1984). A gene for $tRNA_2^{Lys}$ has been cloned and sequenced (Del Rey et al., 1982). This gene contains a 23 base pair intervening sequence identical in sequence to the intron of precursors isolated from *rna1* mutants at nonpermissive temperatures. The number of copies of the $tRNA_2^{Lys}$ gene in the haploid yeast genome has not been established. Southern analysis of yeast genomic DNA using labelled pre-$tRNA^{Ile}$ indicates there are two copies of the gene for this pre-tRNA. These genes for pre-$tRNA^{Ile}$ have been cloned and sequenced (Ogden et al., 1984). Although the anticodons are the same, the two genes differ by a single base pair in the region encoding the TψU loop. Each gene encodes a 60-base pair intron of identical sequence. Surprisingly, there is considerable conservation of sequences in the 5' flank to a position of -38.

There is little sequence homology among the introns of pre-tRNAs of *S. cerevisiae*. However, they do share several common features. The IVS interrupts the coding sequences in the same relative position, one nucleotide 3' to the anticodon. The lowest free energy calculations (Ogden et al., 1984) and solution structure probing (Swerdlow and Guthrie, 1984; and Lee and Knapp, 1985) suggest a common general structure among pre-tRNAs, organized into two domains. These are an exon domain, where the sequences found in the acceptor stem, D arm and the TψC arm are folded into a conventional tRNA structure, and an IVS-containing domain forming an extension of the anticodon stem. In all cases there is a sequence in the intron which is complementary to a portion of the anticodon loop. The splice sites are located at either end of the anticodon-intron helix. The 5' splice site is usually, but not always found in a single-stranded region, whereas the 3' splice site is always in a single-stranded region.

Although the introns in these precursors vary in length from 14 to 60 nucleotides, and do not have any consensus splicing sequences conserved within the introns, or within the mature coding sequences, there are two sites in the introns which do seem to share some homology. In yeast, an A or a U is always found within the intron adjacent to the 3' splice junction. A guanosine is found in the antepenultimate position (-3 from the 3' splice site) in 80% of a total of 33 precursors compared from *S. cerevisiae*, other fungi, plants, *Dictyostelium*, *Drosophila* and vertebrate sources (Szekely et al., 1988). Comparison of the sequences from *S. cerevisiae* that have been published (Ogden et al., 1984; and Stucka and Feldman, 1988) reveals that 6/10 of the sequences have a guanine present in this position. Point mutations at this position were found to affect splicing, although there was no specific requirement for guanosine (Szekely et al., 1988).

Transcription of tRNA genes:

Transcription of eukaryotic tRNA genes by RNA polymerase III is controlled by an internal promoter. Internal deletions and substitutions in a variety of tRNA genes have defined two domains, *box A* and *box B*. These boxes are located in the highly conserved D and TΨC arms, respectively, of the tRNA genes. The *box A* is always located a constant distance from the transcription start site. However, in natural yeast genes distances between *boxes A* and *B* vary from 31 to 93 base pairs. This variation is due to differences in the lengths of the extra arms and the presence of intervening sequences within certain tRNA genes (reviewed in Geiduschek and Tocchini-Valentini, 1988). Studies where the distances between the *A* and *B boxes* have been changed show that separations of approximately 30 - 60 base pairs are optimal (Raymond and Johnson, 1983; Fabrizio et al., 1987; and Baker et al., 1987).

Transcription of tRNA genes by RNA polymerase III requires two transcription factors, TFIIIB and TFIIIC (Lassar et al., 1983; and Burke and Söll, 1985). TFIIIC binds specifically to tRNA genes in the absence of TFIIIB, but is not itself sufficient to activate specific transcription by RNA polymerase III (Klemenz et al., 1982; and Lassar et al., 1983). TFIIIB becomes sequestered into stable tRNA gene transcription complexes only in the presence of bound TFIIIC, suggesting that protein-protein contacts are essential for further complex assembly (Lassar et al., 1983; and Klekamp and Weil, 1986). The binding of transcription factors to tRNA genes occurs in two stages. In the first stage, TFIIIC binding of the *A* and *B boxes* occurs rapidly and is relatively unstable. In the second stage, binding of TFIIIB in the presence of TFIIIC, is slow, temperature dependent and is sensitive to ionic strength (Stillman et al., 1985). The resultant complex is stable for many rounds of transcription. Recently it was shown that yeast TFIIIC functions as an assembly factor for TFIIIB by dismantling already assembled transcription complexes (Kassavetis et al., 1990). TFIIIC can be specifically removed from these complexes by heparin or high ionic strength. TFIIIB alone correctly positions RNA polymerase III for repeat cycles of transcription on tRNA genes with the same efficiency as fully assembled transcription complexes.

Genomic footprinting studies have revealed that TFIIIB forms extended interactions with the 5' flanking DNA of a least some tRNA genes. The 5' flanking region of a $tRNA_3^{Leu}$ gene was protected from DNase I digestion, extending from within the coding region of the gene to about -40, and this protection was dependent on functional *A* and *B box* internal promoters (Huibregtse and Engelke, 1989). This pattern of protection is also seen for $tRNA^{Gln}$, *SUP4* $tRNA^{Tyr}$ and *SUP6* $tRNA^{Tyr}$ (Kassavetis et al., 1989). These results are not surprising in light of the gathering evidence that 5' flanking sequences can modulate tRNA gene expression. The influence of 5' flanking regions on efficient *in vitro* transcription of tRNA genes has been demonstrated for some tRNA genes of *Bombyx mori*, *S. cerevisiae*, *D. melanogaster*, *Xenopus* and humans (Sprague et al., 1980; Larson et al., 1983; Johnson and Raymond, 1984; Dingermann et al., 1982; Hipskind and Clarkson, 1983; Carbon and Krol, 1991; Arnold and Gross, 1987; and Arnold et al.,1988). In yeast, the four genes for $tRNA^{Leu}$, which have been cloned and sequenced, all contain a pentanucleotide sequence, TTTCAACAAATAAGT in their 5'-flank. The replacement of this 15 base pair conserved sequence of a yeast $tRNA_3^{Leu}$ gene with plasmid sequences results in a tRNA gene which is active in *Xenopus* extracts, but nearly inert in yeast extracts (Johnson and Raymond, 1984). This pentanucleotide sequence modulates the expression of yeast $tRNA_3^{Leu}$ *in vivo* as well as *in vitro* (Raymond et al., 1985). In *S. cerevisiae* many tRNA genes are preceded by a $(Py)_3CAACAAA(N)_{\sim 5}$ sequence (Raymond et al., 1985). Whether this sequence modulates the transcription of all the tRNA genes, which it precedes, is not yet known.

Transcription termination elements for RNA polymerase III have only been partially characterised. In yeast, a minimum of six T residues are required for efficient termination (Allison and Hall, 1985). When the T_7 cluster at the end of a yeast tyrosine tRNA gene is shortened to T_5, significant read-through transcription occurs both *in vivo* and *in vitro*. If the T_7 cluster is shortened to T_4, termination is completely abolished. Sequences surrounding the T tract also affect termination. A cluster of 4 of more T residues surrounded by GC-rich sequences on the noncoding strand acts as the signal to terminate transcription at the end of 5S RNA genes of *Xenopus* (Bogenhagen and Brown, 1981). The sequence context of seemingly identical T tracts can affect their usage in termination of transcription (Mazabraud et al., 1987).

RNA polymerase III purified to 90% homogeneity from ovarian tissue of *Xenopus laevis* did not support accurate initiation of transcription at the start site of cloned 5S RNA genes, but did recognise the conserved termination sequences both accurately and efficiently. It was concluded that all the information required for termination of transcription resides in the ability of RNA polymerase III to identify a simple nucleotide sequence at the end of the gene (Cozzarelli et al., 1983). However, observations that the simple termination signal and/or its 3' flanking sequences are protected by components present in transcription factor fractions, some 3' deletion mutants compete poorly for the transcription apparatus, and seemingly equivalent consensus sequences behave differently in various sequence environments all suggest that auxiliary transcription termination factors exist. The La protein may be such a factor (Gottlieb and Steitz, 1989a and b). This phosphoprotein is found associated with all nascent RNA polymerase III transcripts isolated from mammalian cells. It selectively binds to a 3' uridylate stretch encoded by the termination signal and its presence can be detected in active transcription complexes. Immunodepletion leads to diminution of transcriptional activity and shortening at the 3' end of the transcript by one or two residues. Adding back purified La protein to the immunodepleted extracts leads to a partial restoration of transcription activity. The La protein appears to be a release factor for transcription termination, that is required for completion of synthesis and release of the nascent transcript. Recently, an *in vivo* approach has been developed to identify the gene products required for utilization of a transcription termination signal. The selection system, in yeast, uses ochre suppressors weakened by altered transcription termination signals to identify mutations in genes encoding proteins involved in termination of transcription by RNA polymerase III. The first mutation selected, *ret1-1*, is in a gene encoding a previously uncloned subunit of the RNA polymerase III (James and Hall, 1990). It is hoped that this approach will also identify other genes whose products are also required as termination factors for efficient and accurate termination for polymerase III, if such factors are indeed required in *S. cerevisiae*.

End-processing of precursor tRNA transcripts:

The biosynthesis of eukaryotic tRNA involves the removal of 5' leader and 3' trailer sequences, addition of the -CCA_{OH} tail, splicing of intervening sequences, nucleotide modification and transport from the nucleus to the cytoplasm.

The removal of 5' leader sequences from precursor tRNA transcripts (pre-tRNAs) is catalysed by ribonuclease P (RNase P), an endoribonuclease. Most of the information about RNase P derives from work with *E. coli* and *Bacillus subtilis* (reviewed in Pace and Smith, 1990). The prokaryotic RNase P holoenzyme consists of one protein subunit and a RNA subunit. Temperature sensitive mutants show that both are required *in vivo*, however the RNA component alone can recognise and cleave substrates under the appropriate conditions *in vitro*. This enzyme recognises its cleavage site through interaction with the helix formed by the coaxial stacking of the tRNA acceptor stem and the common (TψC) arm. It appears to release the 5' leader sequence from the pre-tRNA by removing a proton from a properly positioned oxygen, triggering an attack by that oxygen on the electrophilic phosphorus in the phosphodiester bond, breaking that bond and resulting in products with 5'-phosphate and 3'-hydroxyl groups.

The nuclear RNase P from *S. cerevisiae* has been purified (Lee and Engelke, 1989). This enzyme, like its prokaryotic counterpart, has an RNA component. This was shown by pretreating purified RNase P with proteinase K or micrococcal nuclease and then testing for residual activity. The RNA has been isolated from this enzyme and sequenced (Lee and Engelke, 1989). This information was used to clone the gene for the RNA component of RNase P (Lee et al., 1991a,b). The RNA alone was incapable of cleaving several eukaryotic and prokaryotic pre-tRNAs under any conditions tested (Lee and Engelke, 1989). This is consistent with work of others on the eukaryotic RNA component of RNase P. The RNA component of RNase P from *S. cerevisiae*, *S. pombe*, and humans can be folded into cage-shaped structures that are very similar to the large domain in the prokaryotic RNase P consensus structure (Forster and Altman, 1989). The structural similarities suggest that the RNase P from eukaryotes and prokaryotes share a common ancestor and function.

Mutations in tRNA genes have been isolated which reduce efficiency of 5' end maturation (Mattoccia et al., 1983; Traboni et al., 1984; Willis et al., 1984; Pearson et al., 1985; Willis et al., 1986; Leontis et al., 1988; and Nichols et al., 1988). These mutations all disrupt the tRNA secondary or tertiary structure. This suggests that the RNase P recognises the overall tRNA conformation and conserved features of tRNA structure rather than specific sequences. Equilibrium binding studies involving specific gel retardation of RNase P-precursor tRNA complexes showed that wild type and mutant tRNA had very similar dissociation constants. It was also shown that the RNA component of RNase P was not necessary for binding, by pretreating the enzyme with micrococcal nuclease. This suggests the protein component of the eukaryotic RNase P may play a role in substrate binding (Nichols et al., 1988).

In *E. coli*, the 3' trailer sequence of pre-tRNA is removed by an exonuclease (RNase D). However, in eukaryotes, the 3' trailer sequence appears to be removed by an endonuclease. This probably occurs after the 5' leader sequence has been removed. Oligonucleotides of the expected sequence for the 5' leader and the 3' trailer can be isolated from pre-tRNA processed in *Drosophila* Kc cell extracts, indicating that the removal of both the 3' trailer and the 5' leader occurs by a single endonucleolytic cleavage (Frendeway et al., 1985). Results of previous studies from *X. laevis* oocytes and yeast support this conclusion (Garber and Gage, 1979; Hagenbuchle et al., 1979; and Melton et al., 1980). Frendeway et al. (1985) were also able to show that the 5' leader sequence is removed before the 3' trailer oligonucleotide, using partially purified 5' and 3' processing activities from *Drosophila*. The 3' processing activity only works on pre-tRNA which has been 5' end processed.

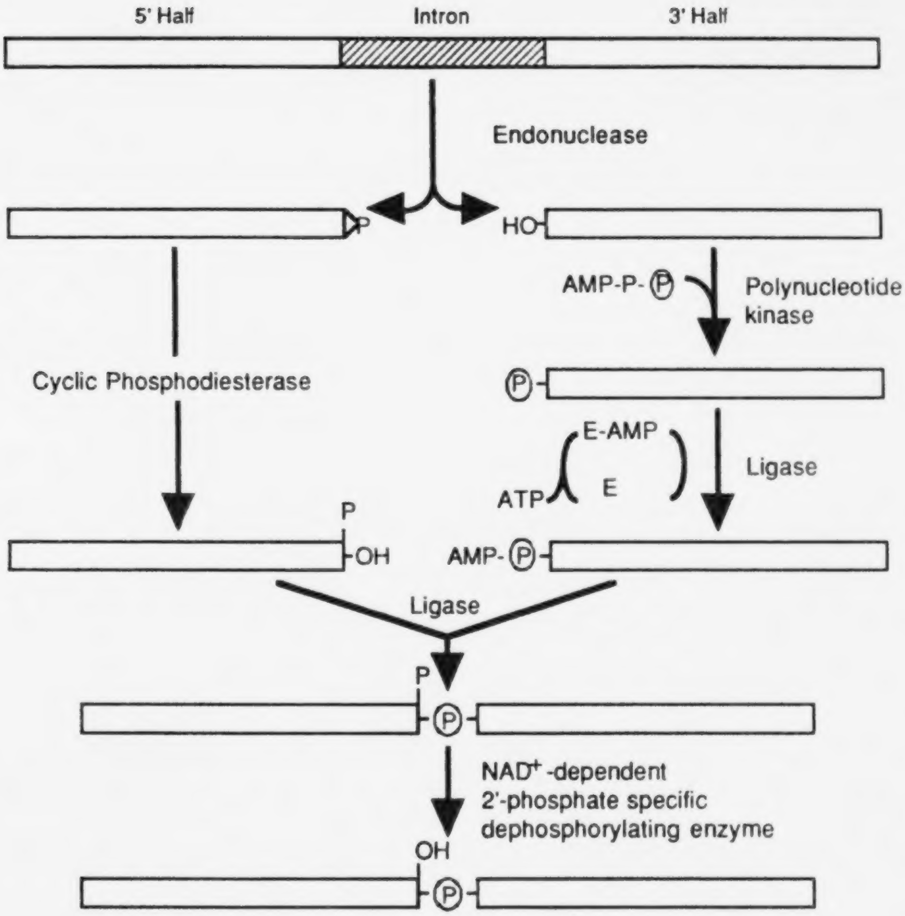
All transfer RNAs have the sequence -CCA_{OH} at their 3' end, the attachment site for the amino acid. In eukaryotes the -CCA sequence must be added post-transcriptionally. This sequence is added after 3' trailer removal, probably before intron removal, by tRNA nucleotidyltransferase (Melton et al., 1980). The 3' -CCA sequences of mature tRNAs are constantly removed and replaced or repaired in the cell (Solari et al., 1977). As expected from this, tRNA nucleotidyltransferase activity can be detected in both the cytoplasm and the nucleus (Melton et al., 1980). The gene for the *S. cerevisiae* tRNA nucleotidyltransferase has been cloned and characterized biochemically (Aebi et al., 1990; and Chen et al., 1990).

Intron-removal from pre-tRNA:

The introns of eukaryotic pre-tRNAs are removed by an RNA splicing mechanism different from group I, group II, or pre-mRNA splicing (reviewed in Culbertson and Winey, 1989). Intron removal was thought to follow end processing (Knapp et al., 1979). However, recent evidence suggests splicing *in vivo* may occur either before or after end trimming, at least for certain pre-tRNAs (O'Connor and Peebles, 1991; and Lee et al., 1991a). Intron removal occurs in two stages which can be distinguished by their requirement for ATP (Figure I-1; Peebles et al., 1979). In the first stage, endonucleolytic cleavage occurs in the absence of ATP resulting in the generation of cleavage products with 5' hydroxyl and 2',3'-cyclic phosphodiester termini (Peebles et al., 1983). The second stage

FIGURE I-1:

Pre-tRNA splicing mechanism in *Saccharomyces cerevisiae*. The tRNA splicing endonuclease cleaves at the 5' and 3' ends of the intron resulting in the release of the intron and generation of a 5' tRNA half with a 2',3'-cyclic phosphodiester terminus and a 3' tRNA half with a 5' hydroxyl terminus. Transfer RNA ligase has polynucleotide kinase, 2',3'-cyclic phosphate 3'-phosphodiesterase, and tRNA ligase activities, and is responsible for joining the tRNA half molecules. The 2' phosphate at the splice junction is subsequently removed by a NAD^+-dependent, 2'-phosphate specific dephosphorylating enzyme. (Adapted from McCraith and Phizicky, 1990).


5' Half
Intron
3' Half
Endonuclease
P
HO
AMP-P-Ⓟ
Polynucleotide kinase
Cyclic Phosphodiesterase
Ⓟ
E-AMP
ATP
E
Ligase
P
OH
AMP-Ⓟ
Ligase
P
Ⓟ
NAD+ -dependent
2'-phosphate specific
dephosphorylating enzyme
OH
Ⓟ

requires the presence of ATP for ligation of the tRNA half molecules to yield mature-sized products with a 2'-phosphate at the splice junction (Greer et al.,1983). This 2'-phosphate is not found in mature tRNA. Its removal is presumably necessary to generate fully functional tRNA.

The cleavage of pre-tRNA at two positions to release the intron and the subsequent ligation of the half molecules are catalyzed by separate enzymes located in the yeast nucleus. The endonuclease and ligase activities of yeast extracts can be physically separated, and the enzymes have quite different physical properties. The endonuclease remains tightly associated with a particulate fraction and can not be solubilized by salt extraction. However, the activity can be solubilized in nonionic detergent (Peebles et al., 1983). These results suggest the tRNA endonuclease is located in the nuclear membrane. The ligase activity is found associated with a rapidly sedimenting fraction from which it can be eluted with high concentrations of salt (Greer et al., 1983). These results are consistent with the tRNA ligase being a soluble matrix or peripheral membrane protein. Although the endonuclease and ligase activities are separable *in vitro*, these enzymes probably act in concert *in vivo*. This conclusion is based on a ligase competition assay, in which the ability of either yeast tRNA ligase or T4 ligase plus kinase to join the tRNA halves produced by endonuclease was measured. These assays demonstrated the preferential access of yeast tRNA ligase to the endonuclease products, providing evidence for the assembly of a functional tRNA splicing complex *in vitro* (Greer, 1986). These two enzymes are sufficient to carry out splicing of all nine of the different intron-containing pre-tRNAs (Peebles et al., 1983; and Greer et al., 1983).

The tRNA endonuclease and ligase have been purified to near homogeneity, and a NAD-dependent, 2'-phosphate specific dephosphorylating enzyme, which can remove the 2'-phosphate left over at the splice junction after removal of the intron and ligation, has been partially purified. The tRNA endonuclease has been purified >5000-fold from a crude Triton X-100 extract of yeast total membranes. This enzyme is a heterotrimer consisting of $\alpha\beta\gamma$ subunits of 31 kDa, 42 kDa and 51 kDa respectively (Rauhut et al., 1990). A gene, *SEN2*, has been isolated which encodes the β subunit of the endonuclease (Rauhut et al., 1990). A mutant allele of this gene, *sen2-3*, accumulates pre-tRNA splicing 2/3 intermediates consisting of the 5' exon covalently joined to the intron (Ho et al., 1990). The *sen2-1* and *sen2-2* mutations result in thermolabile endonuclease activity *in vitro*, but neither allele confers a growth defect *in vivo* (Culbertson and Winey, 1989). The tRNA ligase is a single polypeptide of 95 kDa which has polynucleotide kinase, 2',3'-cyclic phosphodiesterase and tRNA ligase activities organized into discrete domains (Apostol et al., 1991). The amino-terminal sequence of the purified protein was determined and then used to isolate the structural gene from a library of yeast DNA (Phizicky et al., 1986; Westaway et al., 1988). Indirect immune fluorescence confirms that this enzyme is localized in the cell nucleus, and is found primarily at the inner membrane of the nuclear envelope. In cells where the tRNA ligase gene is being overexpressed, staining of the nuclear envelope remains constant. However, the amount of staining in the nucleoplasm increases. This indicates that there are a limited number of ligase sites at the nuclear envelope. It has been hypothesized that the endonuclease constitutes the site for the interaction of ligase with the nuclear envelope (Clark and Abelson, 1987). The 2'-phosphate at the splice junction is highly resistant to nonspecific phosphatases, however, there is a very specific dephosphorylating enzyme capable of removing this phosphate in crude extracts of yeast cells. This crude extract can be resolved into two components required for activity which appear to be distinct from the endonuclease and the ligase (McCraith and Phizicky, 1990). Component one of the tRNA 2'-phosphorylating activity appears to be NAD^+. Dephosphorylation of tRNA bearing a 2' phosphate occurs by transfer of the 2' phosphate to an unidentified acceptor molecule (McCraith and Phizicky, 1991).

The majority of mutations which affect the removal of the intron from pre-tRNAs are found in the mature coding sequences (Baldi et al., 1983; Mattoccia et al., 1983; Winey et al., 1986; and Mathison et al., 1989). Drastic alterations can be made to the sequence of the intron without affecting intron removal from pre-tRNA (Raymond and Johnson, 1983; Strobel and Abelson, 1986a; and Reyes and Abelson, 1988). Mutations in the intron which do prevent splicing of the IVS from the pre-tRNA either eliminate the loop at the 3' splice site, or destabilize the secondary structure of the entire precursor (Raymond and Johnson, 1983; Strobel and Abelson, 1986b; and Szekely et al., 1988). These results, in conjunction with the conserved location of the introns and the common structure of the precursor, support a model in which the tRNA splicing enzymes interact with the sequences in the mature domain of the tRNA (Greer et al., 1983). Further evidence for this model comes from studies showing that ligase specifically crosslinks to bases in the region of the 3' splice site of pre-$tRNA^{Phe}$ (Tanner et al., 1988). Since the addition of ligase to endonuclease incubations stimulates cleavage in an ATP-independent manner, and ligase specifically binds to pre-tRNA in the absence of endonuclease (Apostol and Greer, 1991), the model has been refined to propose that tRNA ligase binds newly synthsised and partially modified pre-tRNA, and then escorts the precursor to the nuclear membrane where a ternary complex is formed with endonuclease. Splicing then takes place within this complex, and the spliced tRNA is transported across the nuclear membrane into the cytoplasm (Tanner et al., 1988; and Apostol and Greer, 1991).

Auxiliary proteins involved in intron removal from pre-tRNA:

Mutations in several genes have identified proteins which appear to play an auxiliary role in tRNA splicing. The first of these genes found was *RNA1* (Hutchinson et al., 1969). The *rna1-1* allele is a temperature sensitive mutation which exhibits pleiotropic defects in RNA processing. The best characterized defect is the accumulation of pre-tRNAs that are highly modified and have mature 5' and 3' termini but contain intervening sequences (Hopper et al., 1978). In an attempt to determine how the *RNA1* gene product can affect the diverse processes involved in RNA maturation, structural analyses of the wild type and mutant *RNA1* genes were performed (Traglia et al., 1989). Although it is now known that the *RNA1* gene encodes a hydrophilic protein of 407 amino acids, the biochemical basis of the defects in RNA processing remains elusive.

A mutant allele of another gene, *los1-1*, was selected using conditional loss of suppression as an assay (Hopper et al., 1980). The *los1-1* mutation causes an accumulation of end trimmed but unspliced precursor tRNAs at the nonpermissive temperature. Mutants in the *LOS1* gene are capable of splicing precursor tRNAs, but apparently at a slower rate than wild type cells. The *LOS1* gene has been cloned and analysed at a molecular level (Hurt et al., 1987). This gene is present in a single copy in the haploid yeast genome. Disruptions and deletions of *LOS1* have been engineered. Haploid yeast cells carrying these disruptions have similar phenotypes to cells that have the *los1-1* allele. Thus, the *LOS1* product promotes efficient precursor utilization at elevated temperatures, but is not required in splicing *per se*. Mutant alleles of two additional genes, *PTA1* and *TPD1*, also result in the accumulation of end trimmed but unspliced pre-tRNAs (O'Connor and Peebles, cited in Culbertson and Winey, 1989; and van Zyl et al., 1989).

Mutations in a fifth gene, *SEN1*, result in accumulation of end trimmed but unspliced pre-tRNAs. *SEN1* was isolated in a screen for mutations that affect endonuclease activity (Winey and Culbertson, 1988). Strains with the *sen1-1* mutation are temperature sensitive for growth, accumulate end-matured intron containing tRNA precursors *in vivo* and have reduced *in vitro* tRNA endonuclease activity. The *SEN1* gene has been cloned and is currently being investigated with genetic and immunological techniques (Culbertson and Winey, 1989). *SEN1* is a single-copy, essential gene. The protein product of this gene, detected by Western blotting, is 140 kDa. This protein is, thus, unlikely to be a component of the tRNA endonuclease, however it may be a positive effector of the tRNA endonuclease.

The *STP1* gene was identified in a screen for yeast DNA sequences that specifically enhanced the expression of the processing-defective *SUP4 (G37)* tRNA. Some but not all intron containing tRNA precursors accumulate in a *stp1* null background. It has consequently been proposed that *STP1* encodes a tRNA species-specific product that functions as a helper for pre-tRNA splicing (Wang and Hopper, 1988).

Nucleotide modifications in tRNA:

Transfer RNAs contain a diverse array of modified bases (reviewed in Bjork et al., 1987). Presently more than 50 different modified nucleotides have been characterized. All modifications are derivatives of the normal nucleotides. Synthesis of modified nucleotides occurs at the polynucleotide level after transcription of tRNA genes with the exceptions of queuosine and inosine. The two latter modifications occur by direct base replacement.

The nucleotide immediately 3' to the anticodon is highly prone to be modified, and a correlation exists between the kind of modification present at this position and the codon recognition properties of the tRNA (Bjork et al., 1987). For example, isopentenyladenosine (i^6A) occurs in tRNAs at position 37 adjacent to the 3' end of the anticodon and is found only in tRNAs that recognize codons starting with U (McCloskey and Nishimura, 1977). The tRNA of a *mod5-1* mutant has 1.5% of the normal tRNA complement of i^6A. The efficiency of suppression by a tyrosine-inserting UAA nonsense suppressor is reduced in a *mod5-1* genetic background. However, the tRNA from this mutant accepts the same levels of amino acid *in vitro* as fully modified species. The loss of i^6A apparently affects the function of tRNA in protein synthesis at a step subsequent to aminoacylation (Laten et al., 1978). Modifications at this position have generally been thought to influence the efficiency and accuracy of the tRNA in its decoding function. In addition, it has been suggested modification of this base may be involved in reading frame maintenance (Bjork et al., 1987). Interestingly, $tRNA^{Phe}$ normally contains a wybutosine (yW) modification at position 37. A yW base-deficient hypomodified $tRNA^{Phe}$ isoacceptor donates its phenylalanine preferentially to globin in competition with the fully modified yW-containing $tRNA^{Phe}$ (Smith and Hatfield, 1986). Modifications at this position do not always improve the efficiency of tRNA in translation.

The wobble position of the anticodon (34) is also highly prone to be modified. Yeast tRNAs that read split codon families have 5-methoxycarbonyl methyl-2-thiouridine (mCm^5S^2U) or 5-amino methyluridine (nm^5U) modifications (Bjork et al., 1987) at position 34. The presence of these nucleotide modifications has been suggested to explain why yeast ochre suppressors do not read amber nonsense codons, as their bacterial counterparts do (Sherman, 1982). Inosine is found exclusively in tRNA where U, C, and A in the third position of the codon all specify the same amino acid (Elliot and Trewyn, 1984). Inosine in the wobble position should allow base pairing with uridine, adenine and cytosine, thus expanding the codon recognition potential (Smith and Hatfield, 1986). Deletion of the intervening sequence from the suppressor allele of the gene encoding $tRNA^{Leu}$ results in a significant reduction in suppressor activity in comparison to the unaltered suppressor alleles. The reduction in suppressor activity correlates with the absence of a 5-methylcytosine modification of the anticodon wobble base

(Strobel and Abelson, 1986a). Modification of the wobble base in the anticodon can have profound effects on the spectrum of codons that a given tRNA can read, and these modifications play a role in the efficiency with which a tRNA can recognize the appropriate codons.

Modifications at other positions in the anticodon can also affect the efficiency of the tRNA in its decoding role. This was demonstrated for yeast $tRNA^{Tyr}$. The $tRNA^{Tyr}$ normally has a pseudouridine modification at the middle anticodon position. This modification is absent in mature tRNA transcribed from a gene from which the intron has been deleted. Suppressor tRNA transcribed from an intron-less gene has a reduced suppression efficiency relative to suppressor tRNA transcribed from an unaltered gene. The absence of the pseudouridine modification probably accounts for the reduction in suppressor efficiency (Johnson and Abelson, 1983).

Four yeast genes involved in modification of tRNA have been identified, *MOD5*, *TRM1*, *TRM2* and *MIA*. *MOD5* has been cloned and sequenced (Dihanich et al., 1987; and Najarian et al., 1987). This gene complements the N^6-(Δ^2-isopentenyl)-2-methlythio-adenosine deficiency of the *miaA E. coli* mutant providing strong evidence that *MOD5* is the structural locus for an enzyme required to make the i^6A modification (Najarian et al., 1987). The *trm1* mutant is defective in the enzyme activity necessary to methylate guanosine to

N^2,N^2-dimethylguanosine (Phillips and Kjellin-Straby, 1967). Mutations in *trm2* result in a deficiency for tRNA (uridine-5) methyltransferase. The tRNA from *trm2* mutants lack methyluridine or ribothymidine (Hopper et al., 1982). Mutations in either *trm1* or *trm2* affect the synthesis of mitochondrial and cytoplasmic N^2,N^2-dimethylguanosine and 5-methyl-uridine, respectively. Neither of these mutations have an obvious physiological defect. The *TRM1* gene has been cloned, using genetic complementation as a positive selection assay. Expression of this gene in *E. coli* converts the organism from one that, neither makes N^2,N^2-dimethylguanosine, nor has N^2,N^2-dimethylguanosine specific tRNA methyltransferase activity into one which does. This provides strong evidence that *TRM1* is the structural gene for tRNA N^2,N^2-dimethylguanosine methyltransferase (Ellis et al., 1986). As was observed for the *trm1* and *trm2* mutants, *mia* mutants have no obvious physiological defect either. The tRNAs in a mutant *mia* background are lacking some of the dihydrouridine moieties. Some but not all tRNAs are affected by *mia*. This suggests that there may be more than one enzyme involved in the formation of the dihydrouridine modification in tRNA (Lo and Bell, 1981; and Lo et al., 1982).

Unmodified $tRNA^{Phe}$ transcripts can be produced by run off transcription. Melting profiles of native and unmodified $tRNA^{Phe}$ suggest that the unmodified transcripts have a less stable tertiary structure, however unmodified $tRNA^{Phe}$ is aminoacylated with virtually normal kinetics (Sampson and Uhlenbeck, 1988). The availability of a completely unmodified tRNA makes it possible to evaluate the role of the modifications in the structure and function of the molecule by adding modifications one by one and examining their effects.

Overall modifications in tRNA do not appear to be essential for viability, but they do seem to fine tune tRNA activity.

Aminoacylation of tRNA:

Transfer RNA plays an essential role in translation by acting as an adaptor molecule which can provide an amino acid to the growing polypeptide chain in response to its corresponding codon. Before a tRNA molecule can act as an adaptor it must be charged with its cognate amino acid. The specificity of decoding resides in complementary base-pairing between the anticodon of the tRNA and the codon of the mRNA. This was demonstrated in a classical experiment where Cys-tRNA^{Cys} was reduced with Raney nickel to give Ala-tRNA^{Cys}. Alanine was then incorporated in response to codons for cysteine in a cell-free system (Chapeville et al., 1962). Therefore, the maintenance of accurate decoding conferred by the specific codon-anticodon interaction is absolutely dependent on the charging of the tRNAs with their correct cognate animo acids. The aminoacyl-tRNA synthetases (AARs) are responsible for this process.

Recent experiments have suggested that tRNA discrimination could be studied by changing a relatively small part of the tRNA sequence. Initially it was shown that a leucine-specific amber suppressor tRNA could be transformed into an amber suppressor tRNA recognized by the serine AAR by altering twelve bases in the acceptor stem, and the D stem and loop (Normanly et al., 1986b). This approach has now been used to determine the minimal number of nucleotide substitutions (identity set) that can alter the amino acid identity of a tRNA for a number of tRNAs. From these studies it has become evident that there are different mechanisms for tRNA discrimination by the AARs. The AARs for the *E. coli* tRNA$^{\mathrm{Leu}}$, tRNA$^{\mathrm{Ser}}$, and tRNA$^{\mathrm{Ala}}$ recognize identity sets found in the acceptor stem and D loop of the mature tRNA (Normanly et al., 1986b; Hou and Schimmel, 1988; McClain and Foss, 1988a; McClain et al., 1988; Rogers and Söll, 1988; Franklyn and Schimmel, 1989). In contrast, the anticodons of *E. coli* tRNA$^{\mathrm{fMet}}$, tRNA$^{\mathrm{Met}}$, tRNA$^{\mathrm{Trp}}$, tRNA$^{\mathrm{Ile}}$, tRNA$^{\mathrm{Val}}$, yeast tRNA$^{\mathrm{Val}}$, and beef tRNA$^{\mathrm{Trp}}$ are the major identity sets these tRNAs (Knowlton et al., 1980; Muramatsu et al., 1988; Schulman and Pelka, 1988; Pallanck and Schulman, 1991). For several tRNAs of *E. coli* (tRNA$_2^{\mathrm{Gln}}$, tRNA$^{\mathrm{Arg}}$, and tRNA$^{\mathrm{Phe}}$) and tRNA$^{\mathrm{Phe}}$ from yeast, major identity determinants have been mapped to both the anticodon and the acceptor stem (Chakraburtty, 1975; McClain and Foss, 1988b; McClain and Foss, 1988c; Sampson et al., 1989; Normanly et al., 1990; Jahn et al., 1991; Pallanck and Shulman, 1991). Thus, the tRNAs encoded by suppressor alleles may have an altered identity. For example, tRNA$^{\mathrm{Trp}}$ suppressors in *E.*

coli insert glutamine in response to the appropriate nonsense codon (Soll, 1974; and Yaniv et al., 1974).

Clearly tRNA sequence is important for recognition and aminoacylation by a cognate aminoacyl-tRNA synthetase. The accuracy of aminoacylation *in vivo* also depends on the balance of tRNA and aminoacyl-tRNA synthetase. Overexpression of *E. coli* Gln-tRNA synthetase leads to the incorrect acylation of *supF*, an amber suppressor tRNA^{Tyr}, with glutamine. If $\text{tRNA}_2{}^{\text{Gln}}$ is also overexpressed, incorrect aminoacylation of tRNA^{Tyr} is no longer observed (Swanson et al., 1988). Most glutamine-aminoacyl synthetase and tRNA^{Gln} molecules may normally be complexed with each other *in vivo*. The K_m of the glutamine aminoacyl-synthetase for tRNA^{Gln} is about $0.1\mu M$ whereas both the enzyme and the tRNA are normally present at approximately micromolar concentrations *in vivo*. There is probably little free glutamine-aminoacyl synthetase in the cell. If cellular glutamine-aminoacyl synthetase levels are increased without a concomitant increase in the tRNA^{Gln} levels, then uncomplexed synthetase molecules are free to interact with noncognate tRNA species, which could then lead to mischarging. Thus, the balance of *in vivo* complex formation between tRNA and its cognate aminocyl-tRNA synthetase contributes significantly to the fidelity of aminoacylation.

Multiple species of tRNA exist for a single amino acid, but there appears to be only one aminoacyl-tRNA synthetase for each amino acid. Many of these enzymes have been characterized (reviewed in Schimmel, 1987). In spite of the common catalytic features of these enzymes, the proteins are widely diverse in their subunit sizes and quaternary structures. They range in size from 300 - 1000 amino acids and are found in various quaternary forms. Interestingly, the quaternary structure and subunit size of at least some of the enzymes is conserved throughout evolution. In spite of the apparent diversity, the AARs can be classified into two classes based on common structural features (Cusack et al., 1990). The proteins of the first class have a dinucleotide-binding fold based around a five-strand parallel β sheet. These AARs also share the so-called HIGH region, a his-ile-gly-his motif. In the second class of AARs the active site is formed on top of the central part of a seven-stranded antiparallel β sheet.

Construction of suppressor alleles of a *S. cerevisae* tRNA^{Trp} gene:

The original goal of this thesis was to construct a functional opal suppressor allele of a *S. cerevisae* tRNA^{Trp} gene, which could be used in studies on suppression in *D. melanogaster.* However, the project evolved into a study of the processing of suppressor alleles of the tRNA^{Trp} gene in yeast. Chapter II of this thesis discusses the construction of a functional opal suppressor using site-directed mutagenesis. It was found that two changes, one in the anticodon, and one in the intron of the gene, were required to construct an active suppressor. Intron-containing precursors of the gene with a single change in the anticodon appear to accumulate in transformants. This observation led to the proposal that base-pairing at the 3' splice junction of transcripts from the gene with the single change prevents efficient pre-tRNA processing. In Chapter III, experiments testing this hypothesis are presented. Additional changes were made in the anticodon and the intron of the $tRNA^{Trp}$ gene which were expected to either inhibit or encourage base-pairing at the 3' splice junction of the precursor. Chapter IV attempts to address the question: Why have suppressor alleles of the *S. cerevisiae* $tRNA^{Trp}$ gene not been found by conventional techniques? The copy number of $tRNA^{Trp}$ genes in the *S. cerevisiae* haploid genome and the chromosomal locations of these genes were determined. The genes were cloned by PCR and sequenced. A copy of one of the genes was replaced with the *URA3* gene in a diploid and the products of sporulation analysed. These experiments do not answer the question, but they do raise some intriguing possibilities.

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CHAPTER II: Construction of an opal suppressor by oligonucleotide directed mutagenesis of a *Saccharomyces cerevisiae* tRNA^{Trp} gene[1]

INTRODUCTION

A compilation of the primary sequences of *Saccharomyces cerevisiae* tRNA precursors which contain intervening sequences has been published (Ogden et al., 1984). The introns in these precursors are of variable length and do not have any consensus splicing sequences conserved within the introns, or within the mature coding sequences. Two common features are present, however. The introns are all located at the same position, interrupting the anticodon loop one base 3' to the anticodon, and they contain sequences which are complementary to the anticodon. The conservation of the position of the intron and the lack of conserved consensus sequences suggest a common structural basis for recognition by the tRNA splicing enzymes.

The structural requirements for intron removal have been investigated. Suppressor alleles can be isolated from intron-containing genes encoding tRNA^{Ser}, tRNA^{Tyr} and tRNA^{Leu} by conventional genetic techniques (Etcheverry et al., 1982; Goodman et al., 1977; and Andreadis et al., 1982). Suppressor alleles of these genes with introns no longer have sequences in the intron complementary to the anticodon. However, since these alleles encode functional suppressors, the complementarity between the anticodon and the intron must not be essential for intron removal. When the sequences of the pre-tRNAs containing introns (Ogden et al., 1984) are compared, the majority of conserved bases are found in the mature coding sequences and are important in stabilizing the secondary and tertiary interactions in the tRNA product. This has led to a model in which the endonuclease interacts with the sequences in the mature domain of the tRNA precursor. The conserved location of the intron ensures the correct alignment of the splice junctions with respect to the endonuclease (Greer et al., 1983).

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Support for this working model for intron removal has come from studies in which drastic alterations have been made to the intron. It was shown (Johnson et al., 1980) that insertion of a 21 bp fragment into the 32 bp intron of a $tRNA_3^{Leu}$ gene did not inhibit the excision and splicing of the precursors or alter the site of the junction. It was subsequently shown that other extensive alterations could be made to the intron of this gene without affecting intron removal (Raymond and Johnson, 1983; Strobel and Abelson, 1986a and b). Alterations to the intron which do affect the removal of the introns from tRNA precursors either eliminate the loop at the 3' splice site, or destabilize the secondary structure of the entire precursor (Raymond and Johnson, 1983; Strobel and Abelson, 1986a and b; Szekely et al., 1988). In contrast to the scarcity of mutations in the intron which affect intron removal, many mutations have been isolated in the mature coding sequences of *S. cerevisiae* tRNA genes which affect removal of the intron (Baldi et al., 1983; Mathison et al., 1989; Mattoccia et al., 1983; and Winey et al., 1986). An elegant study (Reyes and Abelson, 1988) demonstrated the importance of conserved bases in the mature domain of pre-tRNA^{Phe} for removal of introns, by showing that changing these bases can have a strong effect on endonuclease recognition of pre-tRNA^{Phe}. In particular, the length of the anticodon stem and loop determines splicing specificity, and a purine is required at the 5' side of the 3' splice junction. The structure and sequence of the intron had little effect on splice site selection. Studies of *Schizosaccharomyces pombe* tRNA transcript processing in *S. cerevisiae* also implicate exon mutations as causes of deficient splicing activity (Greer et al., 1987; and Pearson et al., 1985).

The first example of a yeast suppressor tRNA constructed from a tRNA^{Trp} gene of *S. cerevisiae* was recently described (Kim and Johnson, 1988). *In vitro* mutagenesis was used to alter the anticodon of a *S. cerevisiae* tRNA^{Trp} gene from CCA to CTA, and the product of this putative suppressor gene was an efficient and specific suppressor of amber nonsense mutations. The same approach did not create suppressor tRNAs which insert tryptophan in response to opal and ochre codons. The results of others (Dingerman et al., 1988) demonstrate that removal of the 13 bp intron from precursor transcripts of the *Dictyostelium discoidium* $tRNA^{Trp}$ genes in *S. cerevisiae* can only occur if minimal base-pairing of the anticodon with intron sequences is possible. This suggests that the previous attempt to obtain ochre and opal suppressors may have failed (Kim and Johnson, 1988) because the potential for anticodon-intron base-pairing in the transcripts was disrupted. It was possible to create a specific opal suppressor from a $tRNA^{Trp}$ gene of *S. cerevisiae* (shown herein) by altering the sequence in the intron of the $tRNA^{Trp}$ gene, containing a previously engineered UCA anticodon, to restore conventional base-pairing potential between the intron and the anticodon of precursor transcripts of this gene. Herein, evidence is presented that intron-containing precursors tend to accumulate in yeast strains transformed with suppressor constructs having only the requisite anticodon change, and it is speculated that this accumulation is due to an altered secondary structure of the precursor which inhibits splicing at the 3' splice junction.

MATERIALS AND METHODS

Materials:

Restriction enzymes and other DNA modifying enzymes were purchased from BRL and Pharmacia, and were used according to the manufacturer's instructions. Radioisotopically labelled compounds were obtained from New England Nuclear or ICN. Oligonucleotides were 5' end-labelled with [γ^{32}P]-ATP (4500Ci/mmole), and [α^{32}S]-dATP (500 Ci/mmole) was used for DNA sequencing. Yeast tRNA^{Phe} and *E. coli* 5S RNA were end labelled with [α^{32}P]-pCp (2000 Ci/mmole). The plasmid 2BTrp, containing a yeast tRNA^{Trp} gene (Kang et al., 1980) on a 3.2 kb fragment of yeast DNA, was a gift of G. Knapp. Oligonucleotides were synthesized by the Regional DNA Synthesis Laboratory, University of Calgary, or in the Department of Microbiology DNA Synthesis Facility, University of Alberta. The tRNA^{Phe} from brewer's yeast and the 5S RNA of *E. coli* , used as a size markers in Northern hybridization experiments, were purchased from Boehringer Mannheim.

Bacterial and Yeast Strains:

Escherichia coli HB101 was used as a host to propagate plasmid DNAs and *E. coli* JM105 was used as a host to propagate M13 bacteriophage DNAs. *S. cerevisiae* strains JG113-5R and JG369-3B were kindly provided by J. P. Gelugne. The genotypes of these strains are listed in the footnote to Table II-1. The *S. cerevisiae* strain RJ293-13C (*MATa*, *his4-260*, *ura3-52*, *leu2-3,112*) was kindly provided by J. Johnson. These yeast strains were used to test for suppressor activity of the putative tryptophan-inserting opal suppressors.

DNA Manipulations:

Preparation and storage of plasmid and M13 DNA, restriction analysis, and plasmid subcloning were performed according to standard methodology (Maniatis et al., 1982). Bulk yeast RNA was isolated by phenol extraction, and the tRNA fraction for Northern hybridizations was purified by DEAE-cellulose chromatography (Lo et al., 1982).

Site-Specific Oligonucleotide-Directed Mutagenesis:

A 0.41kb *Hinc*II-*Hae*III fragment of yeast DNA from 2BTrp, which includes the yeast $tRNA^{Trp}$ gene, was subcloned into the *Eco*RI site in M13mp19 using *Eco*RI linkers. The corresponding single-stranded DNA was used as the template for mutagenesis using the double primer method (Stewart et al., 1985).

Yeast transformations:

The 0.41kb *Hinc*II-*Hae*III fragments, with the wild type or modified $tRNA^{Trp}$ gene, were subcloned into YRp17, a high copy number plasmid, and YCp50, a single copy number plasmid. *S. cerevisiae* strains JG113-5R, JG369-3B and RJ293-13C were transformed with these constructs by polyethylene-glycol-induced genetic transformation (Klebe et al., 1983).

Northern Hybridizations:

Yeast tRNA was resolved on 10% polyacrylamide, 8.3 M urea gels and transferred onto Genescreen Plus membranes using the electroblot protocol recommended by the manufacturer (duPont Co.). The Northern blots were hybridized using a standard protocol (van Tol and Beier, 1988), with modifications (Wallace and Miyada, 1987). The blots were prehybridized in 5 X SSC, 5 X Denhardts, 0.01 M phosphate buffer pH 7.0, 0.2 % SDS, 1 mM ATP, 1 mM EDTA and 150 μg/ml denatured salmon testes DNA for 6 hours, at 55°C. Hybridization was overnight, in the same buffer with 1 X 10^6 cpm ^{32}P end-labelled oligonucleotide probe per ml hybridization buffer, at 55°C. After hybridization, the filters were washed five times at room temperature, twice at 55°C for 30 min., and once at 65°C for 10 minutes in 6 X SSC, or until the background counts could no longer be detected with a hand-held geiger counter.

RESULTS AND DISCUSSION

Cloning and Mutagenesis

A single base change in the CCA anticodon of $tRNA^{Trp}$ is sufficient to create tRNAs capable of base-pairing with the opal (UGA) nonsense codon. To determine whether a $tRNA^{Trp}$ with such an altered anticodon would function as an opal nonsense suppressor, oligonucleotide directed *in vitro* mutagenesis was used to change the CCA anticodon of a yeast $tRNA^{Trp}$ to UCA (Figure II-1). The presumed secondary structure of the $tRNA^{Trp}$ precursors transcribed from the wild type and engineered genes is shown in Figure II-2. Since it has been demonstrated that removal of a 13 bp intron from precursor transcripts of the *D. discoidium* $tRNA^{Trp}$ genes in *S. cerevisiae* occurred only if base-pairing of the anticodon with intron sequences is possible (Dingermann et al., 1988), a second change was made in the engineered $tRNA^{Trp}$ gene (Figure II-1). This results in restoration of conventional base-pairing potential between the intron and the anticodon in the precursor product of this gene. The expected secondary structure of this precursor is illustrated in Figure II-2C.

Analysis of the Putative Suppressors In Vivo

The 0.41 kb *Hinc*II-*Hae*III fragments of yeast DNA containing the wild type and mutant forms of the yeast $tRNA^{Trp}$ gene were subcloned into YRp17 and YCp50, using *Eco*RI linkers. The YRp17 constructs were transformed into JG113-5R, JG369-3B and

RJ293-13C. Complementation of the *trp1-1* allele by *TRP1* on the vector was used to select for the presence of the YRp17 constructs in JG113-5R. Complementation of the *ura3-52* allele by *URA3* on the vector was used to select YRp17 transformants of JG369-3B and RJ293-13C. YCp50 transformants of JG369-3B and RJ293-13C were also selected for by complementation of the *ura3-52* mutation. Transformants were replica plated onto selective media to test for suppression of nonsense markers.

The suppressor phenotype of JG113-5R transformed with the wild type and engineered tRNA^{Trp} genes is presented in Table II-1. No suppression was observed for transformants transformed with YRp17 or the construct containing only the wild type tRNA^{Trp} gene, as expected. Suppression of the opal markers, *leu2-2* and *his4-260*, was seen when JG113-5R was transformed with pYRtRNA^{Trp}op2, the plasmid with the tRNA^{Trp} gene that has both the altered anticodon and correspondingly altered intron. These transformants were unable to suppress amber or ochre alleles. Therefore, tRNA^{Trp}op2 is a specific suppressor of opal alleles. Transformants with the plasmid containing the tRNA^{Trp} gene which has only the anticodon change (pYRtRNA^{Trp}op1) were unable to suppress opal alleles in JG113-5R, even after extended incubations of up to two weeks. Thus, both the anticodon and the intron changes are required to obtain a specific opal suppressor from this yeast tRNA^{Trp} gene. The ability to suppress UGA alleles, conferred upon the transformants by the presence of pYRtRNA^{Trp}op2, was confirmed by demonstrating that spontaneous loss of tryptophan prototrophy by plasmid loss resulted in a concurrent loss of the ability to suppress the *leu2-2* and *his4-260* mutations in all cases (results not shown). Therefore, the ability of these transformants to grow in the absense of leucine or histidine results only when a functional opal suppressor tRNA encoded by the altered tRNA^{Trp} gene on the pYRtRNA^{Trp}op2 plasmid is present.

To determine if the suppressor phenotype of the transformants is influenced by plasmid copy number or the genetic background, the YRp17 and YCp50 constructs were transformed into additional *S. cerevisiae* strains, JG369-3B and RJ293-13C. JG369-3B was constructed independently of JG113-5R, but has the same markers as JG113-5R with two additions: *lys2-1*, an ochre suppressible allele and *ura3-52*, a nonsuppressible mutation. RJ293-13C has the suppressible *his4-260* opal allele in a *leu2-3,112*, *ura3-52* background. The suppressor phenotype of JG369-3B transformed with wild type and engineered tRNA^{Trp} genes cloned into YRp17 (a high copy number vector) is also presented in Table II-1. There was growth of the JG369-3B strain, when transformed with the pYRtRNA^{Trp}op2 plasmid, on leucine and histidine omission media within three to five days at 30^{o}C. In this genetic background, tRNA^{Trp}op2 was unable to suppress ochre or amber alleles. Thus, this genetic background did not alter the suppressor phenotype of tRNA^{Trp}op2. The pYRtRNA^{Trp}op2 transformants of RJ293-13C also grew on the histidine omission medium (results not shown), so tRNA^{Trp}op2 can also suppress opal nonsense mutations in this genetic background. No suppression of opal alleles was observed in JG369-3B and RJ293-13C strains transformed with pYCtRNA^{Trp}op2 (a low copy number plasmid) or the pYRtRNA^{Trp}op1 or pYCtRNA^{Trp}op1 constructs, even after prolonged incubation at 30°C. Since the gene for tRNA^{Trp}op2 must be present in a high copy number to observe opal suppression, tRNA^{Trp}op2 must be a relatively weak suppressor. In all three genetic backgrounds tested, both the anticodon change and the intron change are required to obtain an opal suppressor from the tRNA^{Trp} gene. Opal suppression by tRNA^{Trp}op1 was not detected in these genetic backgrounds, possibly because the gene product of the tRNA^{Trp}op1 gene may not be functional as a suppressor, there may be no mature product, or so little mature product that this *in vivo* assay is not sensitive enough to detect a low level of suppressor activity.

An additional surprising result was obtained in both the JG369-3B and RJ293-13C genetic backgrounds. In extended incubations at 30°C of up to two weeks, weak growth was seen for pYRtRNA^{Trp}wt transformants on media selecting for opal suppression, but no suppression was observed on plates selecting for suppression of ochre or amber nonsense mutations. This result was not observed in the JG113-5R genetic background. Thus the wild type tRNA^{Trp} can act as an extremely weak opal suppressor, but only in the genetic backgrounds of JG369-3B and RJ293-13C. The CCA anticodon in wild type tRNA^{Trp} may rarely decode the UGA opal codon by C·A mispairing. There are examples of tRNA's with wild type anticodons which can functionally interact with nonsense codons. A tRNA of *E. coli* that could insert tryptophan at UGA codons has been described (Hirsh, 1971). This tRNA is not mutated in the CCA anticodon, but has a mutation in the D arm. In *S. cerevisiae*, wild type $tRNA^{Gln}{}_{CAA}$ and $tRNA^{Gln}{}_{CAG}$ can suppress opal and amber nonsense mutations respectively, when present on high copy number plasmids (Pure et al., 1985; and Weiss and Freidberg, 1986). Genetic background plays an important role in whether or not the wild type $tRNA^{Trp}$ can act as an extremely weak suppressor, since suppression by the wild type $tRNA^{Trp}$ was only observed in the JG369-3B and RJ293-13C genetic backgrounds. These strains may have a modifier gene(s) which permit the pairing of the C in the first position of the anticodon with the A in the UGA opal nonsense codon. Modifier genes have been selected in *S. cerevisiae* which enhance or decrease suppressor efficiency (reviewed in Sherman, 1982), and others that result in UAG suppression in a UAA suppressor genetic background (Gelugne and Bell, 1988). The latter class of modifier genes may be analogous to the modifier genes we postulate to be present in JG369-3B and RJ293-13C.

Northern Analysis of $tRNA^{Trp}$ Transcripts from the Transformants

Bulk tRNA from untransformed JG113-5R and transformants of this strain was analysed to determine if any changes in transcription or processing of $tRNA^{Trp}$ precursors could be detected.

The Northern hybridization blots were first probed with a 21 base oligonucleotide complementary to the antisense strand of the 3' end of the *S. cerevisiae* tRNA^{Trp} gene. This oligonucleotide will hybridize to all precursors, and the mature product of the yeast tRNA^{Trp} gene. Utilizing this probe, a predominant band was detected that migrated slightly faster than the 77-nucleotide pCp-labelled yeast tRNA^{Phe} (Figure II-3A). Mature yeast tRNA^{Phe} migrated only slightly slower than the 75-nucleotide mature tRNA^{Trp} transcript. Therefore, we feel this predominant band corresponds to the mature yeast tRNA^{Trp} transcript. A band indicating hybridization to trace amounts of tRNA^{Trp} in the yeast tRNA^{Phe} preparation was also observed. The autoradiogram was purposely over-exposed to detect smaller amounts of precursor. Lighter bands, which migrate considerably more slowly, were detected from all bulk tRNA preparations (Figure II-3B). These bands correspond to precursor transcripts and processing intermediates of the tRNA^{Trp} genes. There appear to be increased levels of these transcripts in the yeast transformed with either the wild type or the engineered tRNA^{Trp} genes cloned into YRp17. Yeast replicating plasmids are maintained within the yeast cells at up to 100 copies per cell with an average of several copies per cell (Ausubel et al., 1989). There are six endogenous copies of the tRNA^{Trp} gene in the haploid genome of *S. cerevisiae* (Kim and Johnson, 1988; and this thesis, Chapter IV). Thus, pYRtRNA^{Trp}wt, pYRtRNA^{Trp}op1 and pYRtRNA^{Trp}op2 transformants may have several times as many tRNA^{Trp} genes as the YRp17 transformants and untransformed strains. An increase in the amount of precursor detected in these transformants is, therefore, not surprising. However, pYRtRNA^{Trp}op1 transformants appear to accumulate even more precursor transcripts than either pYRtRNA^{Trp}wt or pYRtRNA^{Trp}op2 transformants (Figure II-3A).

The Northern blots were also probed with an end-labelled 20 base oligonucleotide which is complementary to 10 bases 3' to the intron and 10 bases 5' to the intron in the yeast tRNA^{Trp} gene product. Under the hybridization conditions used, this probe will only recognize transcripts which do not contain the intron. With this probe, only the band which corresponds to the mature tRNA^{Trp} transcript was detected (Figure II-3C). Thus, the precursors which hybridized to the first probe contain intervening sequences. Alteration of the CCA anticodon seems to affect the ability of the yeast cell to splice the intervening sequences out of the precursor tRNA^{Trp} transcripts effectively. Since the accumulation of intron containing precursors in the yeast transformed with pYRtRNA^{Trp}op2 is comparable to the yeast with the extra copies of the wild type tRNA^{Trp} gene (Figure II-3A), the ability to remove the intron from the precursor efficiently is restored when the second change is made in the intron.

There are two alternative secondary structures possible for the anticodon stem, loop and intron of the wild type pre-tRNA^{Trp} (Ogden et al., 1984). In the first conformation (Figure II-4A) the anticodon loop is base paired with a complementary five base sequence in the intron leaving the 3' splice junction in a single strand loop. This secondary structure conforms to the consensus secondary structure suggested for *S. cerevisiae* tRNA precursors. The consensus secondary structure for yeast pre-tRNAs with introns was determined by solution structure probing of pre-tRNA^{Tyr}, pre-tRNA^{Phe}, pre-tRNA^{Pro}, pre-tRNA^{Leu} and pre-tRNA^{Ile}. In each case, sequences in the anticodon loop were found to be base paired with a short sequence in the intron, and the 3' splice junction located in a single stranded loop a constant distance from the 5' splice junction (Lee and Knapp. 1985; O'Farrell et al., 1978; and Swerdlow and Guthrie, 1984). The second possible conformation (Figure II-4B) is very similar to the secondary structure of pre-tRNA^{Pro} (Lee and Knapp, 1985). In this structure the bases from the 3' end of the anticodon loop are base paired with bases from the intron. The 3' splice junction is also single stranded. The free energy of the secondary structure of the anticodon stem, loop and intron of the precursor tRNA^{Trp} in these conformations was estimated using published guidelines (Freier et al., 1986). Both conformations should be stable, however the second structure has a lower free energy than the first and therefore will be more likely to form than the first conformation (Table II-3).

The change in the anticodon from CCA to UCA in tRNA^{Trp}op1 results in a U·G base pair in the anticodon-intron complementary sequence in the first conformation. Although U·G base pairs do not cause distortion in the helical structure, they contribute less to stabilization than the C·G base pair, and U·G base pairs have weaker nearest neighbour interactions (Freier et al., 1986). The secondary structure of op1 in Figure II-5A is likely to be less stable than the conformation in Figure II-5B (Table II-3). In the latter conformation the 5'UUCAAU3' sequence from the anticodon loop could base pair with the underlined bases in the 3'AA^{C}GUUA5' sequence in the intron. The 3' splice site of this structure would now be located in a double stranded region. Mutations in the introns of pre-tRNA$_3^{Leu}$ and pre-tRNA^{Pro}, which reduce or eliminate removal of the intron, have been found to affect the 3' splice site. These mutants all reduce the size of the single stranded loop at the 3' splice junction. Precursors which have a fully double stranded structure at the 3' splice site were not cleaved by the endonuclease, although cleavage could still occur at the 5' splice junction (Raymond and Johnson, 1983; Strobel and Abelson, 1986a; and Szekely et al., 1988). The precursors of tRNA^{Trp}op1 may accumulate because cleavage at the 3' splice junction is inhibited. This would result in no, or very little, mature product and consequently we would be unlikely to detect suppression of the *leu2-2* and *his4-260* opal alleles in the *in vivo* assay system we employed.

The second change made in the tRNA^{Trp}op2 gene restores the potential for conventional base pairing between the anticodon loop and the 5 base pair sequence in the intron. The secondary structure in conformation 1 has a lower free energy and would be expected to be stable (Figure II-5A). This second site change would reduce the stability of conformation 2 (Figure II-B). In this conformation both the wild type pre-tRNA^{Trp} and pre-tRNA^{Trp}op1 have a G in position 22 which could base pair with a C in position 28. In pre-tRNA^{Trp}op2 an A replaces the G in position 22. The free energy of this structure would then be more positive and thus would be less likely to form. Pre-tRNA^{Trp}op2 in conformation 1 would be a suitable substrate for endonucleolytic cleavage of the intron since this conformation places the 3' splice junction in a single stranded region. As a result mature tRNA^{Trp}op2 would be expected. Consequently strains transformed with pYRtRNA^{Trp}op2 should be able to suppress *leu2-2* and *his4-260* opal alleles and growth would be expected on leucine and histidine omission medium, as was observed.

The arguments made above predict that tRNA^{Trp} genes with the anticodon sequence altered from CCA to CTA should encode an amber suppressor tRNA which will be efficiently processed into a mature tRNA. The precursor transcribed from this gene would be relatively unstable in either the first or the second conformations suggesting there is little or no secondary structure in the region of the 3' splice site. This leaves the 3' splice junction located in a single stranded loop and mature transcripts would be expected. This prediction has been tested. It has been reported (Kim and Johnson, 1988) that a single change in the CCA anticodon sequence to CTA is sufficient to create an efficient and specific suppressor of amber nonsense mutations. We have confirmed this result by showing that when the gene for this suppressor tRNA is present on a single copy plasmid colonies are observed on media selecting for amber suppression within one to two days at 30^{o}C, and no suppression of the ochre or opal alleles was observed in either JG369-3B or JG113-5R transformants. However, when yeast cells are transformed with the tRNA^{Trp} amber suppressor gene on a high copy number plasmid they grow very slowly. Thus, this suppressor seems to be toxic when present on a high copy number plasmid, presumably by interfering with the cell's normal chain termination process resulting in reduced cell viability.

This argument also predicts that maturation of transcripts will be inhibited for putative ochre suppressors in which the CCA anticodon sequence of the tRNA^{Trp} gene has been altered to TTA. The anticodon stem and loop, and the intron of this precursor would likely be unstable in conformation 1, but more stable in conformation 2. In the second conformation the UU bases in the anticodon could base pair with the AA sequence flanking the 3' splice junction, thus occluding splicing at this junction. As predicted, this putative ochre suppressor is inactive *in vivo* (Kim and Johnson, 1988; and this thesis, Chapter III). A second change in the 5 base intronic sequence to restore complementarity between the anticodon loop and the intron may result in efficient processing, because the second site changes would reduce the stability of conformation 2 and favour the formation of conformation 1. This, in turn, may result in an active suppressor, if the ochre tester alleles can be suppressed with tryptophan insertion at the premature stop codons. Experiments to test this prediction have been done and are reported in the accompanying chapter III.

FIGURE II-1:

Sequence of the wild type and engineered $tRNA^{Trp}$ genes. The sequences of the wild type gene, an op1 gene ($tRNA^{Trp}$op143), and an op2 gene ($tRNA^{Trp}$op212) were determined by the dideoxy chain termination method. The sequences which were changed in the anticodon and the intron are indicated.

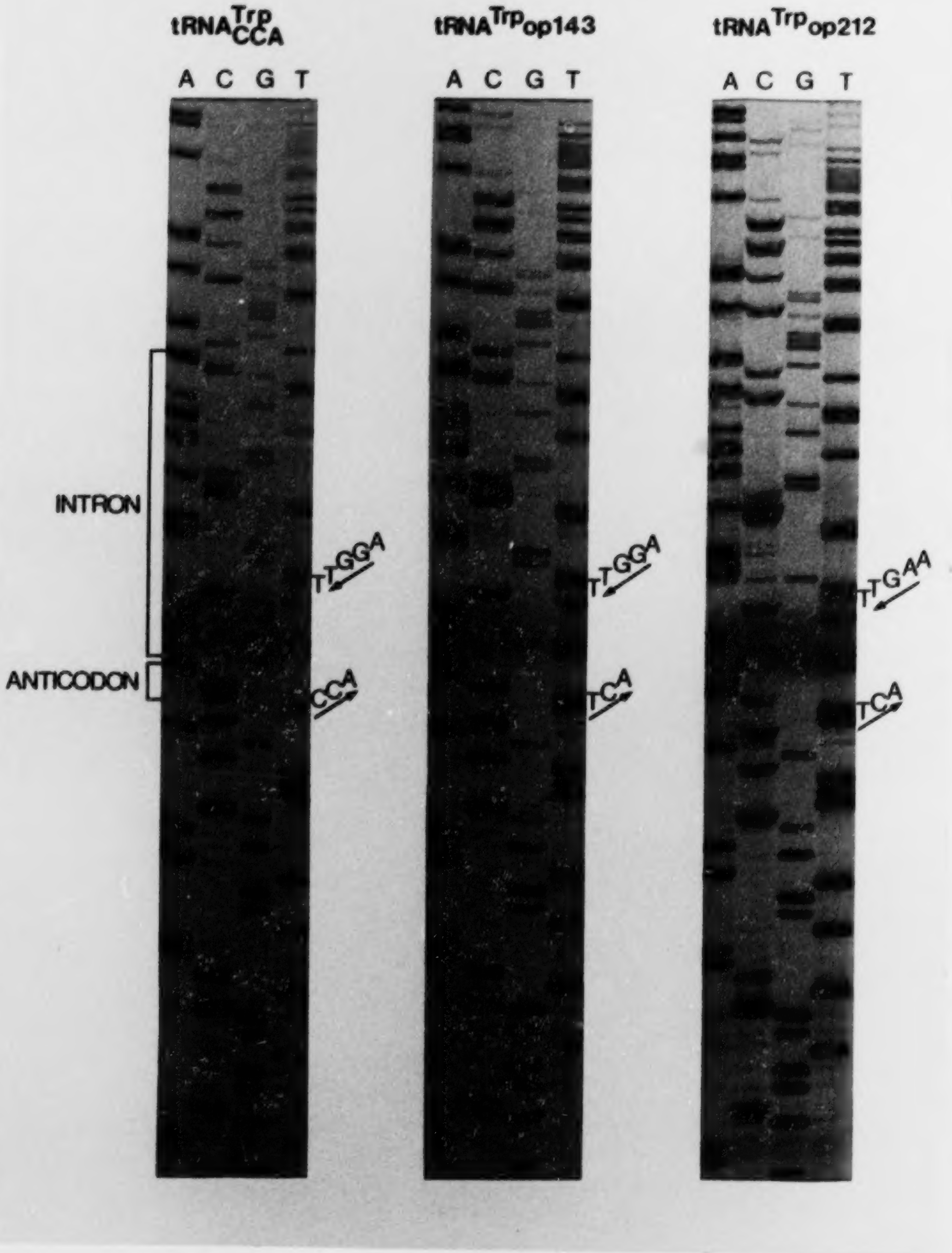

tRNA$^{Trp}_{CCA}$
tRNA^{Trp}op143
tRNA^{Trp}op212
A C G T
A C G T
A C G T
INTRON
ANTICODON
TTGGA
CCA
TTGGA
TCA
TTGAA
TCA

FIGURE II-2:

Cloverleaf structures of wild type and engineered pre-tRNA^{Trp}. The structures conform to the generalized secondary structure of yeast tRNA precursors that have intervening sequences (Ogden et al., 1984). Figure II-2A illustrates the secondary structure of the precursor tRNA^{Trp} encoded by the wild type gene (Kang et al., 1980). The position of the intron with respect to the anticodon and the five bp region of homology between the anticodon loop and the intron is indicated. Figure II-2B shows the possible secondary structure of the precursor tRNA encoded by op1, the tRNA^{Trp} gene which has the single, anticodon change. The region of homology between the anticodon loop and the intron of the precursor tRNA transcribed from this gene is boxed in this figure. The alteration of the tRNA anticodon from CCA to UCA results in a partial disruption of the complementary sequence. Figure II-2C shows the most likely secondary structure of the precursor tRNA transcribed from op2. Op2 has the anticodon change and a change in the intronic sequence from UUGGA to UUGAA, which restores conventional base-pairing potential to the five base pair complementary sequence of the transcripts.

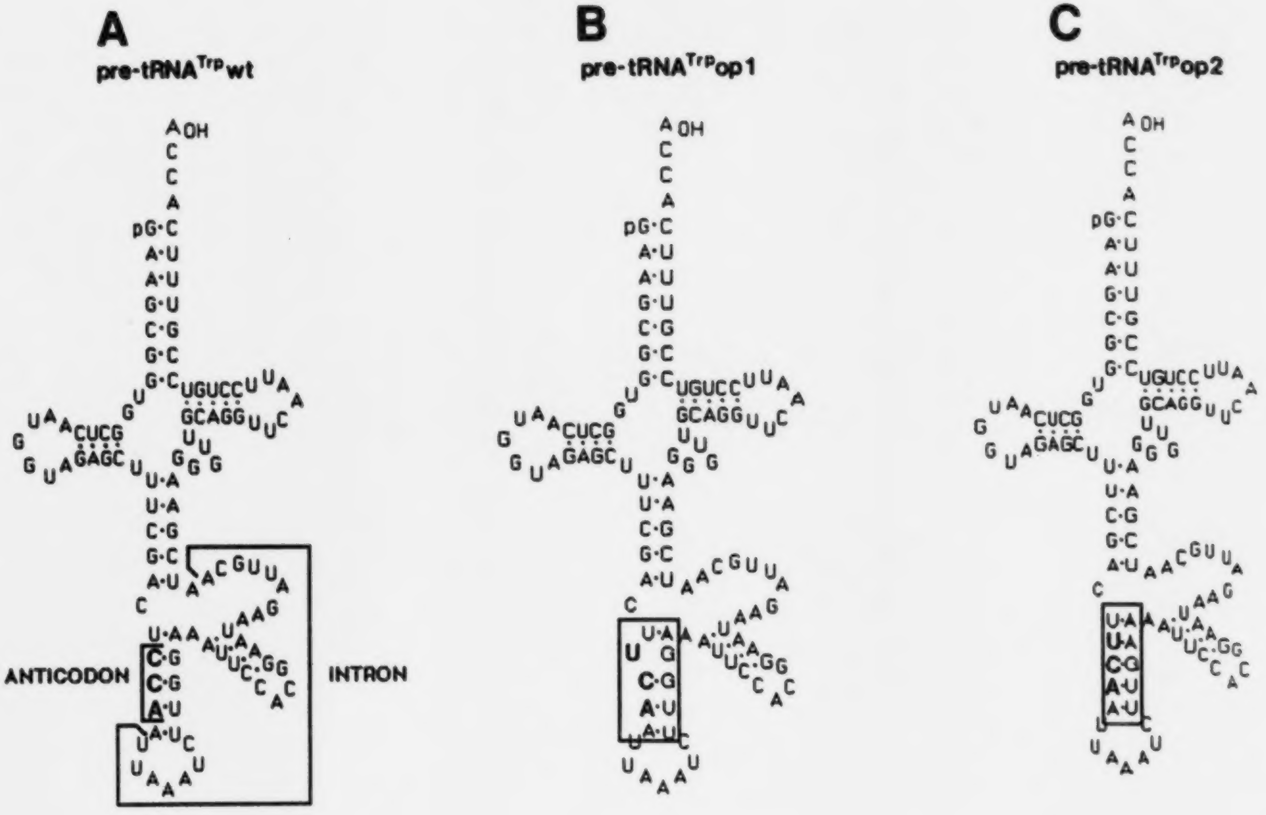

A
pre-tRNA^{Trp}wt
B
pre-tRNA^{Trp}op1
C
pre-tRNA^{Trp}op2
ANTICODON
INTRON

FIGURE II-3:

Northern hybridization analysis of the tRNA$^{\mathrm{Trp}}$ transcripts isolated from the transformants. Five µg partially purified tRNA were electrophoretically resolved on a 10% polyacrylamide, 8.3 M urea gel. Lane 1 was loaded with tRNA isolated from untransformed JG113-5R cells; lane 2, tRNA from JG113-5R transformed with YRp17; lane 3, tRNA isolated from pYRtRNA$^{\mathrm{Trp}}$wt transformants; lane 4, tRNA from pYRtRNA$^{\mathrm{Trp}}$op1; lane 5, tRNA from pYRtRNA$^{\mathrm{Trp}}$op2 transformants; M (left) *Escherichia coli* 5S RNA and yeast tRNA$^{\mathrm{Phe}}$ which were 3' end labelled with pCp(5'-^{32}P). The fastest-migrating band in this marker lane corresponds to the 77-nucleotide end-labelled tRNA$^{\mathrm{Phe}}$. The most slowly migrating band corresponds to the 121-nucleotide end-labelled 5S RNA. In ethidium bromide-stained gels, this band migrates with the yeast 5S RNA. The band which migrates slightly faster than the end-labelled 5S RNA is likely a breakdown product of the 5S RNA. Lane M (right), second marker lane with 1 µg each of unlabelled yeast tRNA$^{\mathrm{Phe}}$ and *E. coli* 5S RNA. Shown are 24 hour (A) and 48 hour (B) exposures of a Northern blot probed with a 5'-end-labelled oligonucleotide complementary to the sequence at the 3' end of the transcripts and a Northern blot (C) probed with a 5' end-labelled oligonucleotide 20 bases in length and complementary to 10 bases in the mature domain immediately 5' to the intron and 10 bases in the mature domain 3' to the intron, under conditions sufficiently stringent that only transcripts which do not contain the intron will hybridize (van Tol and Beier, 1988; and Wallace and Miyada, 1987). The amount of RNA loaded into each lane was monitored by the presence of the 5S rRNA band in ethidium bromide-stained gels. Equal amounts of 5S RNA were observed in each lane in ethidium bromide-stained gels.

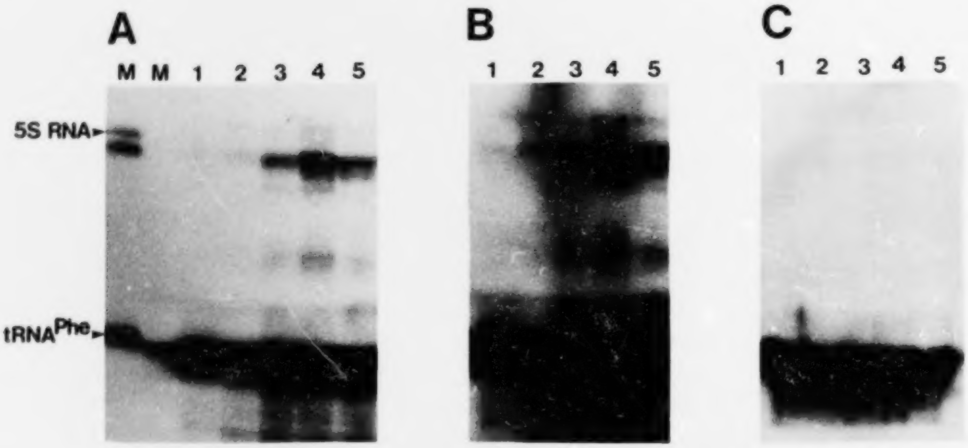

A
M M 1 2 3 4 5
5S RNA
tRNAPhe
B
1 2 3 4 5
C
1 2 3 4 5

FIGURE II-4:

The wild type pre-tRNA^{Trp} may have two alternative secondary structures (Ogden et al., 1984). In conformation 1 (structure on the left), five bases from the anticodon loop are base paired with a complementary sequence in the intron. This structure conforms to the consensus secondary structure for yeast tRNA precursors determined from solution structure probing of pre-tRNA^{Tyr}, pre-tRNA^{Phe}, pre-tRNA^{Pro}, pre-tRNA^{Leu} and pre-tRNA^{Ile} (O'Farrell et al., 1978; Swerdlow and Guthrie, 1984; Lee and Knapp, 1985). The structure on the right illustrates the second conformation which may be possible for pre-tRNA^{Trp}. The intronic sequences are indicated in italic and the anticodon (5'UCA3') is indicated with outlined letters.

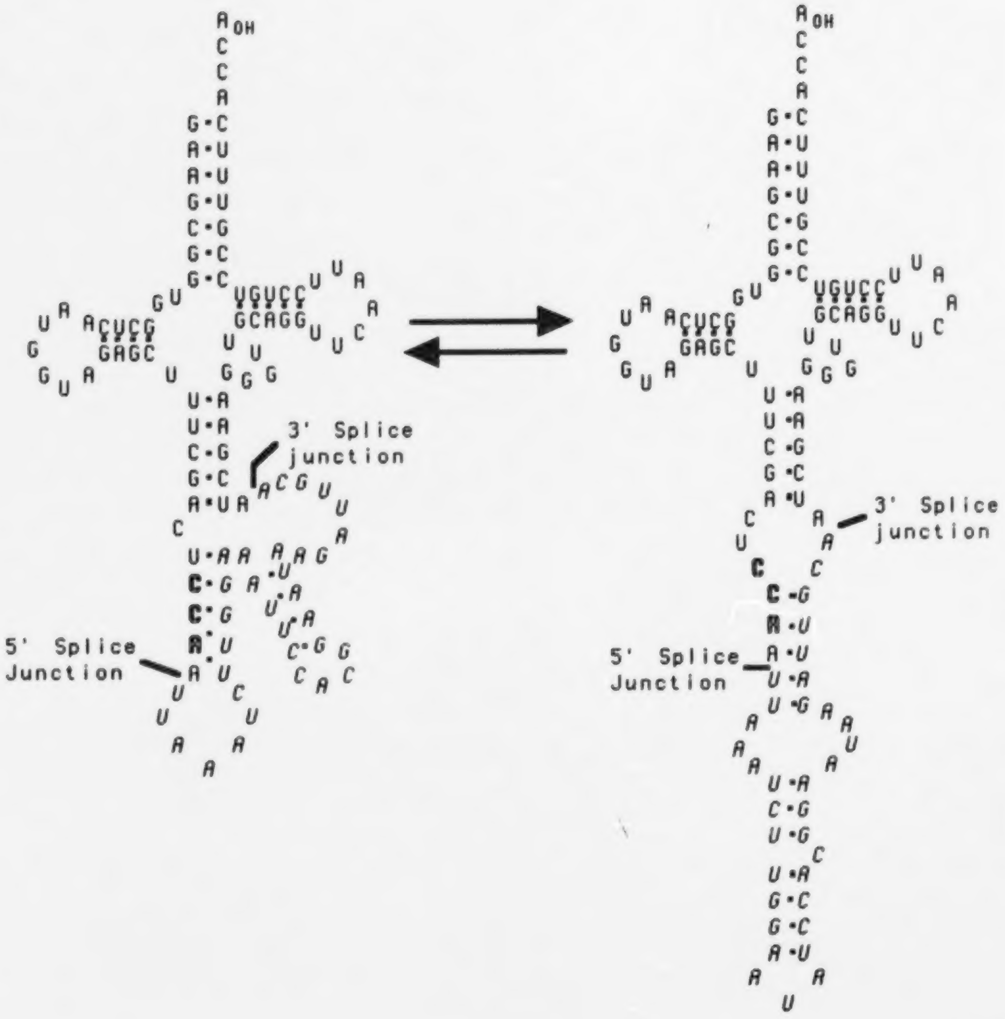
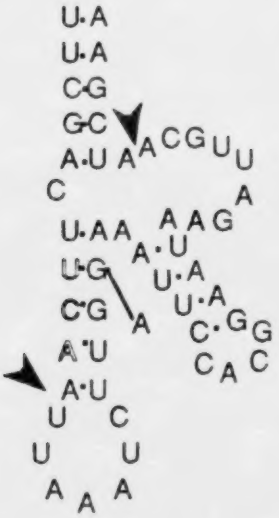

A OH
3' Splice junction
5' Splice Junction
A OH
3' Splice junction
5' Splice Junction

FIGURE II-5:

The alternative secondary structures for the anticodon stem, loop, and the intron of precursors transcribed from op1. The 5' and 3' splice junctions are indicated by bold, filled-in arrowheads. The change made in the op1 gene to restore conventional base-pairing between the anticodon loop and the intronic sequence (in op2) is indicated. The anticodon (5'UCA3') is indicated with outlined letters.


U·A
U·A
C·G
G·C
A·U A ACGUU
C A
U·A A AAG
U·G A·U
C·G A U·A
A·U U·A
A·U C·G G
U C C A C
U U
A A A

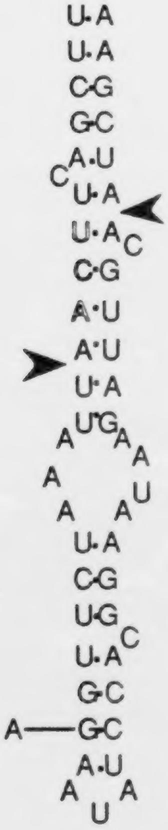

U·A
U·A
C·G
G·C
C A·U
U·A
U·A C
C·G
A·U
A·U
U·A
A U·G A A
A A U
A A
U·A
C·G
U·G C
U·A
G·C
A—G·C
A·U
A U A

TABLE II-1: **Suppressor phenotype of the yeast strains when transformed with wild type and engineered $tRNA^{Trp}$ genes.**

Transformants	Suppressor Phenotype						
	Ochre alleles			Amber alleles		Opal alleles	
	ade2-1	*can1-100*	*lys2-1*	*met8-1*	*trp1-1*[c]	*leu2-2*	*his4-260*
JG113-5R Transformants[a]							
YRp17	-	-	NA[d]	-	NA	-	-
$pYRtRNA^{Trp}$ wt	-	-	NA	-	NA	-	-
$pYRtRNA^{Trp}$ op1	-	-	NA	-	NA	-	-
$pYRtRNA^{Trp}$ op2	-	-	NA	-	NA	+	+
JG369-3B Transformants[b]							
YRp17	-	-	-	-	NA	-	-
$pYRtRNA^{Trp}$ wt	-	-	-	-	NA	-	-
$pYRtRNA^{Trp}$ op1	-	-	-	-	NA	-	-
$pYRtRNA^{Trp}$ op2	-	-	-	-	NA	+	+
YCp50	-	-	-	-	-	-	-
$pYCtRNA^{Trp}$ wt	-	-	-	-	-	-	-
$pYCtRNA^{Trp}$ op1	-	-	-	-	-	-	-
$pYCtRNA^{Trp}$ op2	-	-	-	-	-	-	-

[a] The genotype of JG113-5R is *MATα, ade2-1, can1-100, met8-1, trp1-1, leu2-2, his4-260.*

[b] The genotype of JG369-3B is *MATα, ade2-1, can1-100, lys2-1, met8-1, trp1-1, leu2-2, his4-260, ura3-52.*

[c] The YRp17 plasmid carries the *TRP1* and *URA3* genes. Complementation of the *trp1-1* allele was used to select for the presence of the YRp17 plasmid and it's derivatives in JG113-5R and JG369-3B. Complementation of the *ura3-52* allele was used to select for the transformants of JG369-3B that have the YCp50 plasmid and it's derivatives.

[d] NA indicates not tested. Strain JG113-5R does not have the *lys2-1* allele. The *trp1-1* allele was used to select transformants with YRp17 or its derivatives.

The suppressor phenotype was determined after incubation of 3 to 5 days at 30°C. The minus (-) symbols indicate no suppression was observed within the 3 to 5 day incubation period, and the plus (+) symbols indicates that suppression was observed. See text for unusual results which were observed after extended incubation periods.

TABLE II-2:

Free energies for the secondary structure of the anticodon stem, loop and intron of the precursor tRNA^{Trp} transcripts for each of the two possible conformations.

Transcripts	ΔG_{37} for Conformation 1	ΔG_{37} for Conformation 2
tRNA^{Trp} wt	- 3.3	- 6.1
tRNA^{Trp} op1	+ 0.1	- 2.9
tRNA^{Trp} op2	- 1.3	- 0.5

Calculations of the free energies for the secondary structures in the precursors are based on published methods (Freier et al., 1986).

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CHAPTER III: Processing of precursor transcripts from a *Saccharomyces cerevisiae* tRNA^{Trp} gene may be affected by mutations in the anticodon or in the intron

INTRODUCTION

In *Saccharomyces cerevisiae*, ten tRNA gene families have been identified which contain introns (Ogden et al, 1984; and Stucka and Feldmann, 1988). These genes are transcribed by RNA polymerase III to give precursor transcripts (reviewed by Geiduschek and Tocchini-Valentini, 1988). Maturation of these precursors involves removal of the 5'-leader and 3'-trailer sequences, excision of the intron, modification of specific bases, and addition of the 3' terminal CCA. Removal of the 5'-leader sequences is accomplished by RNase P, which cleaves at a single site to generate the phosphorylated 5'-terminus of the mature tRNA (reviewed in Lee and Engelke, 1989). Processing of the 3'-trailer sequence involves removal of 3' extensions, followed by addition of the terminal CCA sequence by a 3' nucleotidyl transferase (Melton et al., 1980). Splicing of yeast pre-tRNAs may occur before end maturation of the precursor tRNA transcripts *in vivo* (O'Conner and Peebles, 1991). Removal of the intron from precursor tRNA molecules occurs in two distinct steps, distinguished by a requirement for NTP cofactors (Peebles et al., 1979). In the first stage, an endonucleolytic cleavage occurs producing tRNA half molecules and the intervening sequence. Two NTP equivalents are required in the second stage for ligation of the half-tRNA molecules to yield mature tRNAs. The product of ligation is a 3', 5'-phosphodiester linkage, with an extra 2'-phosphate which is subsequently removed by a 2'-phosphotransferase (McCraith and Phizicky, 1990; McCraith and Phizicky, 1991).

For *S. cerevisiae* tRNA genes, the majority of mutations which affect maturation are located within the mature tRNA coding sequences (reviewed in Culbertson, and Winey, 1989). Profound changes can be made to the sequence of the introns in tRNA genes without affecting their removal from pre-tRNA by the splicing complex (Raymond and Johnson, 1983; Strobel and Abelson, 1986a,b; Reyes and Abelson, 1988; and Szekely et al., 1988). However, there are some mutations in intron sequences which are known to affect end maturation or intron removal. Alterations in the intervening sequences can affect the recognition and cleavage of precursor tRNA by RNase P (Leontis et al., 1988). Also, mutations in the intron which either eliminate the loop at the 3' splice site or destabilize the secondary structure of the entire precursor may prevent intron removal (Raymond and Johnson, 1983; and Szekely et al., 1988). A direct test of the importance of a single-stranded loop at the 3' splice junction was done by deliberately inserting sequences within the intron which could base pair with sequences at the 3' splice junction. The resultant end-matured pre-tRNA was shown to have a fully double stranded structure at the 3' splice site by solution structure probing, and these precursors were not cleaved by partially purified tRNA endonuclease (Szekely et al., 1988). Therefore, it appears that although there is no sequence requirement, there is a structural requirement for a single-stranded region at the 3' splice junction for recognition and cleavage at this site by the tRNA endonuclease.

Kim and Johnson (1988) first demonstrated that it is possible to create a specific and efficient amber suppressor by *in vitro* mutagenesis of a *S. cerevisiae* $tRNA^{Trp}$ gene. It has subsequently been shown that a weak but specific opal suppressor can be constructed from the same gene (Kim et al.,1990). Surprisingly, it appears that a $tRNA^{Trp}$ gene with a change in the intron as well as the anticodon is a more efficient suppressor of opal nonsense codons than a gene containing only the requisite anticodon change (Atkin et al., 1990). The accumulation of tRNA precursors in strains transformed with $tRNA^{Trp}$ genes which encode only the altered anticodon suggests this difference in suppressor efficiency may be due to less efficient processing. This may reflect an altered secondary structure of the precursor in which base-pairing at the 3' splice junction inhibits splicing. Herein, an examination of the effect of additional mutations, in the anticodon and in the intron of a *S. cerevisiae* $tRNA^{Trp}$ gene, on the suppressor phenotype of transformants is presented. Whether these mutations affect splicing of the precursor transcripts *in vitro* has also been examined.

METHODS AND MATERIALS

Materials:

Restriction enzymes and other DNA modifying enzymes were purchased from BRL or Pharmacia, and were used according to the manufacturer's instructions. The *S. cerevisiae* tRNA endonuclease used was equivalent to fraction VI of Peebles et al., (1983). Radioisotopically labelled compounds were obtained from ICN or Amersham. Oligonucleotides were 5' end-labelled with $[\gamma^{32}P]$-ATP (4500 Ci/mmole), while $[\alpha^{32}P]$-pCp (2000 Ci/mmole) was used to end-label 5S RNA, $[\alpha^{35}S]$-dATP (500 Ci/mmole) was used for DNA sequencing, and $[\alpha^{32}P]$-UTP (3000 Ci/mmole) was used in the *in vitro* transcription experiments. The plasmid 2BTrp, containing a yeast $tRNA^{Trp}$ gene (Kang et al., 1980), was a gift of G. Knapp. Oligonucleotides for probes or *in vitro* mutagenesis were either purchased from the Regional DNA Synthesis Laboratory, University of Calgary, Canada; synthesized in the Department of Microbiology DNA Synthesis Facility, University of Alberta; or provided by C. L. Peebles. The 5S RNA from *Escherichia coli* (used as a size marker in the Northern hybridization experiments) was purchased from Boehringer Mannheim.

Bacterial and Yeast Strains:

E. coli DH5α was used as a host to propagate plasmid DNAs, and

E. coli JM105 was used as a host to propagate M13 bacteriophage. *S. cerevisiae* strains JG369-3B [*MATα*, *ade2-1*(UAA), *can1-100*(UAA), *lys2-1*(UAA), *met8-1*(UAG), *trp1-1*(UAG), *leu2-2*(UGA), *his4-260*(UGA), *ura3-52*] and JG113-5R [*MATα*, *ade2-1* (UAA), *can1-100* (UAA), *met8-1* (UAG), *trp1-1* (UAG), *leu2-2* (UGA), and *his4-260* (UGA)] were kindly provided by J. P. Gelugne, and were used to test for suppressor activity of the putative tryptophan-inserting suppressors. The *S. cerevisiae* strain JG113-5R was also used to prepare 4S RNA samples for Northern analysis.

DNA and RNA Manipulations:

The preparation and storage of plasmid and M13 DNAs, restriction analyses, and plasmid subclonings were performed according to standard methodology (Sambrook et al., 1989). Bulk yeast RNA was isolated by phenol extraction, and the tRNA fraction for Northern hybridizations was purified by DEAE-cellulose chromatography (Lo et al., 1982).

Site-Specific Oligonucleotide-Directed Mutagenesis:

A 0.41 kb *Hinc*II-*Hae*III fragment of yeast DNA from 2BTrp, which includes a yeast tRNA^{Trp} gene, was subcloned into the *Eco*RI site in M13mp19 using *Eco*RI linkers. The corresponding single-stranded DNA was used as the template for mutagenesis using the double primer method (Stewart et al., 1985).

Yeast Transformations:

The 0.41kb *Hinc*II-*Hae*III fragments, with the wild type or a modified tRNA^{Trp} gene, were subcloned into YRp17 (a high copy number plasmid) and YCp50 (a single copy plasmid). *S. cerevisiae* strains JG369-3B and JG113-5R were transformed with these constructs by polyethylene-glycol-induced genetic transformation (Klebe et al., 1983).

Northern Hybridization:

Five µg samples of partially purified yeast tRNA were resolved on 10% polyacrylamide, 8.3M urea gels and transferred onto Genescreen Plus membranes using the electroblot protocol recommended by the manufacturer (duPont Co.). The Northern blots were hybridized as previously described (Atkin et al., 1990).

In Vitro Transcription and Splicing Assays:

Preparation of labelled, end-matured, intron-containing precursor transcripts and subsequent endonuclease reactions were carried out as described in Szekely et al. (1988), and the products were resolved on 12% polyacrylamide, 8.0 M urea gels. The intron-containing precursors were also resolved on 40% formamide, 7.0 M urea, 12% polyacrylamide gels (Myers et al., 1985). The S2 sequencing gel electrophoresis system was used (Bethesda Research Laboratories).

RESULTS

Construction of opal, ochre and amber suppressor alleles of a *S. cerevisiae* tRNA^{Trp} gene

We and others have shown that opal and amber suppressor alleles can be made from a yeast tRNA^{Trp} gene (Kim and Johnson, 1988; Atkin et al., 1990, and Kim et al., 1990). The amber suppressor which has been constructed by a single change in the anticodon from CCA to CUA is an efficient and specific suppressor of amber alleles. The opal suppressor allele which has a single change in the anticodon is a very weak suppressor of opal alleles. A second change at position 37.11 in the intron of this gene (Figure III-1) produces a more efficient opal suppressor (tRNA^{Trp}op2). These observations led us to believe that the previous suggestion by Ogden et al. (1984) that pre-tRNA^{Trp} has two possible secondary structures may be invoked to help explain these suppressor phenotypes. A change in the anticodon from CCA to UCA can affect the conformation of the precursor and, in turn, the efficiency with which the precursor is cleaved by the endonuclease during intron removal, by allowing base-pairing to occur at the 3' splice junction. The second change in the intron favors the formation of a secondary structure in which the 3' splice junction is again located in a single stranded loop (Atkin et al., 1990).

Three predictions of this hypothesis were tested by introducing additional changes within the anticodon and intron of the tRNA^{Trp} gene (summarized in Figure III-1). First, genes which encode an anticodon that will interact with an opal codon, and which have a second change at the 3' splice junction (U37.34 designated tRNA^{Trp}op2') are expected to produce the same phenotype as tRNA^{Trp}op2, since both contain changes which would prevent base-pairing at the 3' splice junction. Second, we would expect amber suppressor alleles with a single change in the anticodon or those with an additional change in the intron at position 37.10 (tRNA^{Trp}am2) to have the same phenotype, since the amber anticodon is not expected to form a stable base-paired structure with the 3' splice junction. Most of pre-tRNA^{Trp}am1 would be folded into the second conformation. The 3' splice junction should be located in a single stranded loop because stable interactions are unlikely to form between bases at the 3' splice junction and bases in the anticodon loop. Pre-tRNA^{Trp}am2 should fold into conformation 1 and the 3' splice junction will, again, be located in a single stranded loop. Third, the hypothesis predicts that a gene with a UUA (ochre) anticodon (tRNA^{Trp}oc1) would not be processed efficiently unless second changes were made within the intron (positions 37.10 and 37.11; tRNA^{Trp}oc2) or at the 3' splice junction (position 37.34; tRNA^{Trp}oc2') to prevent base-pairing at the 3' splice site. Pre-tRNA^{Trp}oc1 is more stable in the second conformation. In this conformation the U34, U35 will be able to base pair with the A37.34, A38 at the 3' splice junction unless second site changes are made which change the conformation of the pre-tRNA^{Trp} (oc2) or directly interfere with the base pairing at the

3' splice junction (oc2').

In vitro transcription of the engineered tRNA^{Trp} genes and endonucleolytic cleavage of precursor molecules

To examine the effects of the engineered changes on the ability of the endonuclease to cleave at the 5' and 3' splice junctions, the engineered tRNA^{Trp} genes and a *SUP53* control gene were transcribed by a RNA polymerase III extract which is proficient in 5' and 3' end processing and, under the conditions we have used, lacks splicing endonuclease activity (Engelke et al., 1985). These precursor molecules were used as substrate for a partially purified endonuclease (Figure III-2). *SUP53* served as a control for these experiments, since the pattern of products from endonucleolytic cleavage of pre-SUP53 is well defined (Knapp et al., 1979, and Gegenheimer et al., 1983). The migration of tRNA^{Trp} precursor transcripts from each of the genes was the same, except for those from oc2. The precursor molecules transcribed from this gene appear to be migrating faster than the precursors transcribed from the other genes. It has been shown that electrophoresis in gels containing 8M urea is not fully denaturing and that tRNAs may retain some secondary structure (Hegg and Thurlow, 1990). To ensure that the migrational difference observed for pre-tRNA^{Trp}oc2 was not due to residual secondary structure, the precursors were isolated and resolved on a denaturing 40% formamide, 7M urea, 12% polyacrylamide gel (Myers et al., 1985). The migration pattern of the precursors was not altered by the presence of the formamide.

Thus, it is unlikely that pre-tRNA^{Trp}oc2 migrates faster because of a conformational difference. Instead pre-tRNA^{Trp}oc2 is a shorter precursor molecule. This precursor was estimated to be 68 nucleotides in length from three separate experiments.

Pre-*SUP53* was cleaved efficiently into the expected 5' and 3' halves, and intervening sequence (IVS) (Figure III-2A). Pre-tRNA^{Trp}wt produced major bands of the sizes expected for the 5' and 3' halves, and the IVS from this precursor (Kang et al., 1980). There was some degradation of the pre-tRNA^{Trp}op1 and op2 transcripts in the experiment shown; however, in other experiments bands of the expected sizes for the 5' and 3' halves, and the IVS from pre-tRNA^{Trp}op1 and op2 were observed (Figure III-2B). Although both of these precursors were poor substrates for the endonuclease in comparison to the pre-tRNA^{Trp}wt, pre-tRNA^{Trp}op2 appeared to be cleaved slightly more efficiently than pre-tRNA^{Trp}op1. Apparently pre-tRNA^{Trp}op2' is an even better substrate for the endonuclease than the wild type precursor (Figure III-2A). Pre-tRNA^{Trp}am1 and am2 are both cleaved efficiently. Pre-tRNA^{Trp}oc1 and oc2 are poor substrates for endonucleolytic cleavage. While a potential interaction between the ochre anticodon and the 3' splice site in pre-tRNA^{Trp}oc1 predicted this might be a poor substrate, the additional intron changes in pre-tRNA^{Trp}oc2 were designed to allow efficient cleavage by endonuclease (Atkin et al., 1990). As noted above, these intron changes appear to have, unexpectedly, resulted in a shorter precursor and which may have, either directly or indirectly, affected cleavage by endonuclease. Pre-tRNA^{Trp}oc2' was cleaved efficiently by the endonuclease, as predicted.

Phenotype of yeast strains transformed with the engineered tRNA^{Trp} genes

Opal suppressors:

It has been previously reported that a tRNA^{Trp} with a UCA anticodon is a weak suppressor of opal nonsense mutations (Kim et al., 1990). A tRNA^{Trp}op2 is also a weak, but slightly more efficient suppressor of opal nonsense mutations (Atkin et al., 1990). Strains transformed with the tRNA^{Trp}op2' constructs have the same suppressor phenotype as the strains transformed with the tRNA^{Trp}op2 constructs (Table III-1). Suppression was observed when strains were transformed with pYRtRNA^{Trp}op2 or pYRtRNA^{Trp}op2' (high copy number plasmids), but not with pYCtRNA^{Trp}op2 or pYCtRNA^{Trp}op2'(single copy plasmids). Although these suppressors are specific, they are weak. Thus even though pretRNA^{Trp}op2' was more efficiently cleaved by endonuclease *in vitro*, suppressor activity *in vivo* was not increased relative to tRNA^{Trp}op2, suggesting splicing may not be a limiting determinant in suppression by this tRNA.

Amber suppressors:

It has also been reported previously that a tRNA^{Trp} with a CUA anticodon is an efficient and specific suppressor (Kim and Johnson, 1988; Atkin et al., 1990). The hypothesis predicts that processing of the precursor should be efficient with or without the second change within the intron of the gene. This expectation appears to be realized and is reflected in the phenotype of strains transformed with plasmids with the am1 and am2 genes, both of which encode efficient and specific amber suppressors (Table III-1). Am1 and am2 are toxic when present on high copy number plasmids.

Ochre suppressors:

A putative ochre suppressor allele of tRNA$^{\mathrm{Trp}}$ containing the requisite anticodon changes does not suppress ochre nonsense alleles (Kim and Johnson, 1988; Atkin et al., 1990; and Kim et al., 1990). It was expected that a second change in the 5-base intronic sequence that restores complementarity between the anticodon loop and the intron (tRNA$^{\mathrm{Trp}}$oc2), or which decreases complementarity at the 3' splice junction (tRNA$^{\mathrm{Trp}}$oc2') might result in more efficient processing. However, strains transformed with these genes were unable to suppress the ochre nonsense alleles in JG369-3B and JG113-5R (Table III-1).

Northern analysis of tRNA isolated from suppressor-bearing yeast strains

Opal suppressors:

It has previously been shown that yeast strains transformed with pYRtRNA^{Trp}op1 accumulate more precursor tRNA^{Trp} than either the strains transformed with pYRtRNA^{Trp}wt or pYRtRNA^{Trp}op2 (Atkin et al., 1990). Strains transformed with pYRtRNA^{Trp}wt or pYRtRNA^{Trp}op2 appear to have approximately the same amount of precursor; however the pattern of precursor accumulation is not identical (Figure III-3A), suggesting that the intron mutation in this construct may have some effect on splicing. Strains transformed with pYRtRNA^{Trp}op2' accumulate approximately the same amount of precursor with a similar pattern of processing products as strains transformed with the wild type tRNA^{Trp} gene (Figure III-3A). Competitive hybridizations, using a labelled oligonucleotide complementary to nucleotides 25-45, spanning the splice junction, of the mature opal tRNA^{Trp} transcripts and a cold competitor oligonucleotide complementary to the wild type tRNA^{Trp} sequence, were done using the same Northern blots. Autoradiographs of these blots show mature tRNA^{Trp} can easily be detected in strains transformed with pYRtRNA^{Trp}op2 and pYRtRNA^{Trp}op2' (Figure III-3B). Mature tRNA^{Trp}op1 was not detected in these experiments. However, this transcript must be present at least at a low level in these cells since opal suppression has been observed by cells transformed with similar constructs (Kim et al., 1990). The same RNA blots were also probed separately with an end-labelled oligonucleotide complementary to the intron of pre-tRNA^{Ile} as an internal standard. Figure III-3C suggests that lanes 1 to 6 were loaded with the same amount of tRNA.

Amber suppressors:

Strains transformed with pYRtRNA^{Trp}am1 and pYRtRNA^{Trp}am2 accumulate more pre-tRNA^{Trp} than untransformed strains and less tRNA^{Trp} precursor than strains transformed with pYRtRNA^{Trp}wt (Figure III-3A). However, this is not entirely surprising since we and others (Atkin et al., 1990, and Kim et al., 1990) have reported that the presence an amber suppressor allele of a tRNA^{Trp} gene on a high copy number plasmid is toxic. We have found that the pYRtRNA^{Trp}am2 plasmid is also toxic. Therefore, strains with a lower plasmid number, and thus a lower amount of precursor, would be selected. Mature amber tRNA^{Trp} was detected in strains transformed by pYRtRNA^{Trp}am1 or pYRtRNA^{Trp}am2, by competitive hybridization (Figure III-3B). The difference in the amounts of tRNA^{Trp}am1 and tRNA^{Trp}am2 precursor probably reflects the amount of tRNA loaded in each lane (Figure III-3C).

Ochre suppressors:

Yeast strains transformed with pYRtRNA^{Trp}oc1 and pYRtRNA^{Trp}oc2' appear to accumulate more precursor than untransformed yeast strains, and less than pYRtRNA^{Trp}wt transformants (Figure III-3A). The pattern of precursor accumulation in pYRtRNA^{Trp}oc2' transformants appears quite different than that from pYRtRNA^{Trp}wt transformants. This strain appears to be accumulating an excess of an end-extended precursor (O'Connor and Peebles, 1991). Thus end-maturation may be affected by the intron changes in this precursor. This result is in contrast to the efficient processing observed for this precursor in our *in vitro* experiments. Potentially, additional or more stringent requirements exist for processing *in vivo*. The strain transformed with pYRtRNA^{Trp}oc2 accumulates only low levels of pre-tRNA^{Trp}, equivalent to that of untransformed strains (Figure III-3A). This result is consistent with the aberrant processing observed for this precursor in the *in vitro* experiments discussed above. None of the putative ochre suppressor transformants have detectable levels of mature tRNA capable of interacting with ochre nonsense alleles (Figure III-3B). The faint bands observed in lanes 9 to 11 represent weak cross-hybridization of the probe to the wild type transcripts, comparable to that seen in lanes 1 and 3.

DISCUSSION

It has been suggested that there are two possible secondary structures for pre-tRNA^{Trp} (Ogden et al., 1984). Mutations in either the anticodon or the intron will affect the distribution of pre-tRNA^{Trp} in each conformation. We have proposed that the change in the anticodon from CCA to UCA in tRNA^{Trp}op1 results in a pre-tRNA in which base pairing can occur between the anticodon and the 3' splice junction, resulting in accumulation of unspliced precursor. A second change in the intron from A to G at 37.11 allows the pre-tRNA^{Trp} to fold into an alternate conformation in which the 3' splice junction is now located in a single-stranded loop, and as a result mature tRNA^{Trp}op2 would be expected (Atkin et al., 1990). In this paper we have tested this hypothesis by examining the effect of additional mutations in the anticodon and the intron of a *S. cerevisiae* tRNA^{Trp} gene.

If the accumulation of precursor tRNA^{Trp} in pYRtRNA^{Trp}op1 transformants is due to inhibition of splicing by base-pairing at the 3' splice junction, a second mutation in the intron at the 3' splice junction, which prevents base-pairing, should have the same suppressor phenotype as pYRtRNA^{Trp}op2 transformants. The A in the intron of the op1 gene at the 3' splice site was changed to a T (op2'). Strains transformed with pYRtRNA^{Trp}op2' had the same suppressor phenotype as strains transformed with pYRtRNA^{Trp}op2 (Table III-1). In addition, both of these transformants accumulate wild type levels of pre-tRNA^{Trp}, and mature opal suppressor tRNA^{Trp} was detected in the tRNA isolated from these transformants (Figure III-3). These results led us to expect that pre-tRNA^{Trp}op2 and pre-tRNA^{Trp}op2' would be cleaved efficiently by the endonuclease *in vitro*, while pre-tRNA^{Trp}op1 would be a poor substrate. However, it was found that although pre-tRNA^{Trp}op2' was a good substrate, pre-tRNA^{Trp}op1 and pre-tRNA^{Trp}op2 were both cleaved inefficiently by the endonuclease (Figure III-2). The pre-tRNA^{Trp}op2 did appear to be a marginally better substrate than the pre-tRNA^{Trp}op1, which could partially account for its more efficient performance as a suppressor *in vivo*.

Several *S. cerevisiae* genes have been isolated which encode gene products involved in intron removal. One of these, *STP1*, appears to encode a tRNA species-specific product that functions as a helper for pre-tRNA splicing (Wang and Hopper, 1988). This gene was originally isolated as a yeast DNA sequence which specifically enhanced the expression of the *SUP4(G37)* gene; a gene defective in intron removal. Strains with a disruption of *STP1* accumulate intron-containing precursors of some, but not all, families of pre-tRNA. It is possible that efficient splicing of the pre-tRNA^{Trp}op2, most of which would be folded into a different secondary structure than pre-tRNA^{Trp}op2', requires such a gene product and that this gene product is not present in the partially purified endonuclease preparation used in the *in vitro* assays.

Our hypothesis also predicts that amber suppressor alleles (am1 and am2) will have a single stranded 3' splice site and therefore be cleaved by the endonuclease. An amber suppressor allele of a tRNA^{Trp} gene has been shown to be an efficient and specific suppressor of amber nonsense mutations (Kim and Johnson, 1988, and Atkin et al., 1990). Herein, we show that am2 also encodes an efficient and specific amber suppressor (Table III-1), as predicted. Mature amber suppressor tRNA was found in both pYRtRNA^{Trp}am1 and pYRtRNA^{Trp}am2 transformants (Figure III-3B). In addition, both pre-tRNA^{Trp}am1 and pre-tRNA^{Trp}am2 were cleaved efficiently by the endonuclease *in vitro* (Figure III-2).

A change in the CCA anticodon of the *S. cerevisiae* tRNA^{Trp} gene to UUA would encode a pre-tRNA^{Trp}oc1 in which base pairing would be possible between the U33, U34 and the AA sequence at the 3' splice junction. A second site alteration in the intron of the oc1 gene should allow the precursor to fold into a secondary structure in which the 3' splice site is located in a single stranded region. Such mutants were constructed (oc2 and oc2'). None of the ochre suppressor alleles of the tRNA^{Trp} gene were able to suppress the tester ochre nonsense mutations (Table III-1) *in vivo*. Also, mature tRNA was not observed in the tRNA extracts from pYRtRNA^{Trp}oc1, pYRtRNA^{Trp}oc2 or pYRtRNA^{Trp}oc2' transformants (Figure III-3B), which is a sufficient explanation for why suppression was not observed. As predicted by the model, the labeled pre-tRNA^{Trp}oc1 transcript was not cleaved well by the endonuclease *in vitro* (Figure

III-2). Transcripts from oc2 migrated faster than the transcripts from the wild type and other mutant tRNA^{Trp} genes on normal or formamide containing gels, and these transcripts were not cleaved *in vitro* by the endonuclease (Figure III-2). The change in the intronic sequence of oc2 appears to have an unexpected effect on the length of the precursor transcribed from this gene. A shorter than expected transcript could be produced if the changes in the intronic sequence had an effect on the overall secondary structure of the precursor, resulting in aberrant end trimming *in vitro* and probably *in vivo*. It is not surprising then, that strains transformed with pYRtRNA^{Trp}oc2 did not accumulate precursor or mature ochre suppressor. However, transcripts of the oc2' gene were cleaved *in vitro* by the tRNA splicing endonuclease (Figure III-2), but mature ochre suppressor tRNA was not observed *in vivo* (Figure III-3B). In addition, the pYRtRNA^{Trp}oc2' transformants appear to accumulate an excess of pre-tRNA^{Trp} which has not been end matured. It is possible, then, that this precursor can be end-processed and cleaved *in vitro*, but *in vivo* processing requirements may be more stringent and potentially this precursor may be a poor competitor for the enzymes involved in the maturation of the total pool of cellular pre-tRNA.

Mutations in the tRNA^{Trp} gene designed to encourage base pairing at the 3' splice junction were shown to inhibit the processing of pre-tRNA^{Trp} at the intron removal step, while second site mutations in the intron designed to prevent base pairing at the

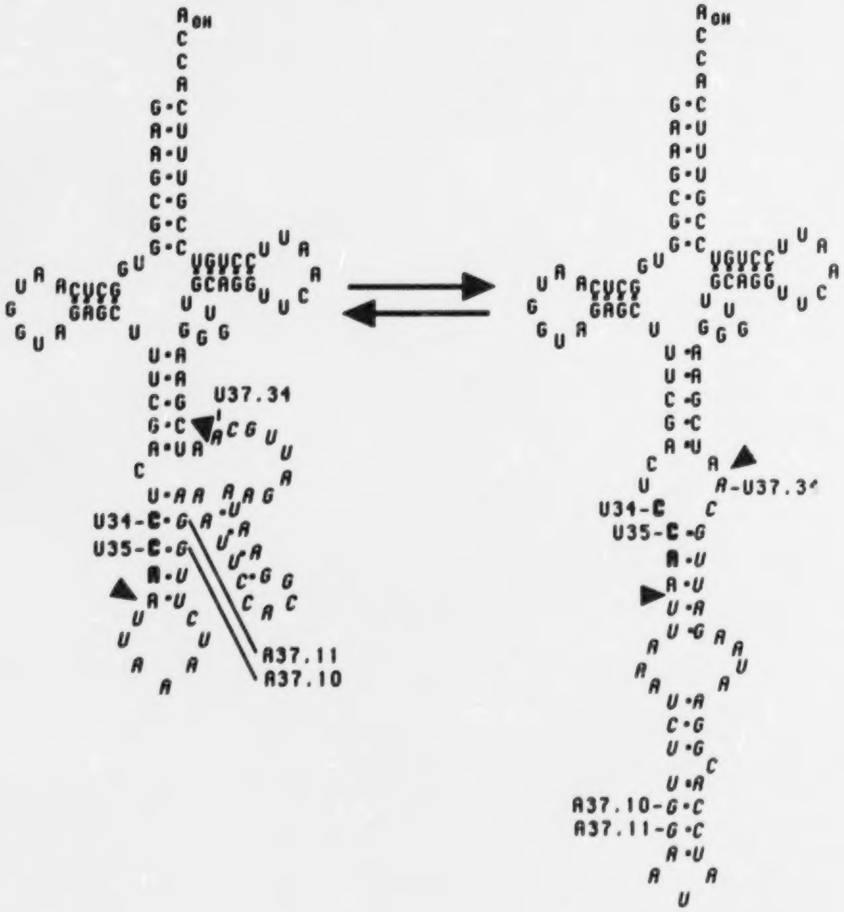
3' splice junction restored recognition and cleavage by the tRNA splicing endonuclease if additional steps in the processing pathway were not thereby disrupted. Experiments are now in progress to characterize the transcript from the oc2 gene, which migrates faster on polyacrylamide gels than those from the other $tRNA^{Trp}$ genes transcribed *in vitro*. These experiments should give us some insight into the nature of the aberrant end trimming, which we speculate must have occurred, to produce the shortened transcript.

To date a functional ochre suppressor allele of a *S. cerevisiae* $tRNA^{Trp}$ gene has not been constructed. As predicted, the anticodon change results in a gene which encodes a precursor from which intron removal is inhibited. However, subsequent attempts to engineer second site mutations within the intron that are predicted to restore intron removal *in vivo* have been unsuccessful. Thus, so far, it is not known whether a *S. cerevisiae* $tRNA^{Trp}$ with a UAA anticodon would suppress ochre nonsense mutations. It may, however, be possible to construct a functional ochre suppressor allele of a yeast $tRNA^{Trp}$ gene by deleting the intronic sequence from a gene that has the requisite change in the anticodon.

FIGURE III-1

Primary sequence and possible secondary structures of the *Saccharomyces cerevisiae* tRNA^{Trp} precursor. (**A**) The sites on the tRNA^{Trp} gene which were altered by site-directed mutagenesis are shown on the two possible secondary structures. Numbering is by the convention established for tRNA^{Phe} (Schimmel et al., 1979). Bold arrowheads indicate the positions of the splice sites. The anticodon is indicated by large open letters, while the intervening sequece is indicated by italics. (**B**) The combinations of changes which were made in the tRNA^{Trp} gene to create the putative opal, amber and ochre suppressor alleles are shown along with the designation for each mutant allele.

A.


U37.34
U34
U35
A37.11
A37.10
A-U37.34
U34
U35
A37.10
A37.11

B.

MUTANT ALLELE	ALTERATIONS CREATED BY SITE-DIRECTED MUTAGENESIS
op1	U34
op2	U34, A37.11
op2'	U34, U37.34
am1	U35
am2	U35, A37.10
oc1	U34, U35
oc2	U34, U35, A37.10, A37.11
oc2'	U34, U35, U37.34

FIGURE III-2

The pattern of cleavage products produced by tRNA splicing endonuclease digestion of gel purified precursors transcribed from the wild type and engineered tRNA^{Trp} genes and the *SUP53* gene. (**A**) Labeled *SUP53* (lane 1), tRNA^{Trp}wt (lane 2), op1 (lane 3), op2 (lane 4), op2' (lane 5), am1 (lane 6), am2 (lane 7), oc1 (lane 8), oc2' (lane 9), and oc2 (lane 10) precursors were prepared by *in vitro* transcription and incubated with yeast tRNA endonuclease. The reaction products were resolved by polyacrylamide-urea gel electrophoresis. (**B**) An autoradiograph from another experiment showing the pattern of products resulting from cleavage by the tRNA splicing endonuclease of tRNATrp wt (lane 1), op1 (lane 2), and op2 (lane 3) precursors.

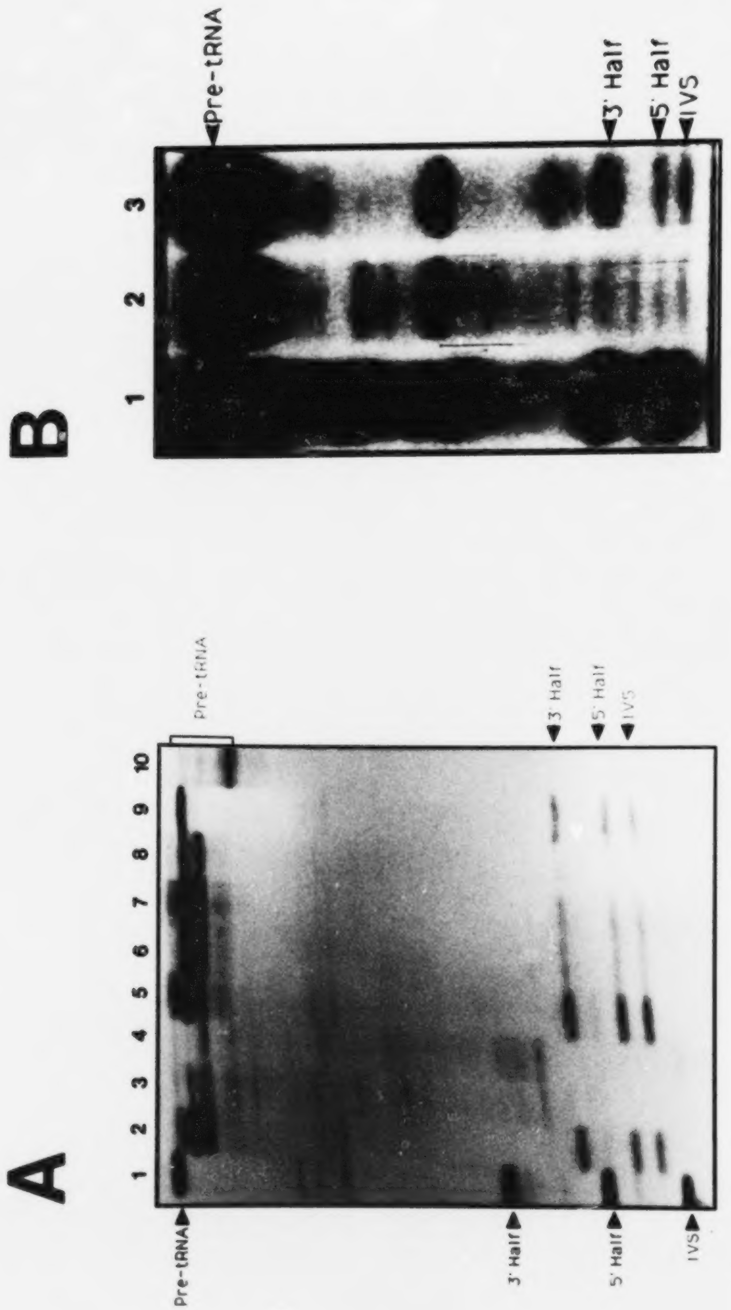

A
1
2
3
4
5
6
7
8
9
10
Pre-tRNA
Pre-tRNA
3' Half
5' Half
IVS
3' Half
5' Half
IVS
B
1
2
3
Pre-tRNA
3' Half
5' Half
IVS

FIGURE III-3

Northern hybridization analysis of the tRNA^{Trp} transcripts isolated from transformants. RNA isolated from untransformed cells (lane 1); cells transformed with YRp17 (lane 2), pYRtRNA^{Trp}wt (lane 3), pYRtRNA^{Trp}op1 (lane 4), pYRtRNA^{Trp}op2 (lane 5), pYRtRNA^{Trp}op2' (lane 6), pYRtRNA^{Trp}am1 (lane 7), pYRtRNA^{Trp}am2 (lane 8), pYRtRNA^{Trp}oc1 (lane 9), pYRtRNA^{Trp}oc2 (lane 10), and pYRtRNA^{Trp}oc2' (lane 11) was resolved on polyacrylamide-urea gels and transferred to GeneScreen plus. (**A**) The tRNA^{Trp} precursors are shown in Northern blots probed with an end-labelled oligonucleotide complementary to 21 bases in the 3' end of the transcripts. The mature tRNA is located below the bottom edge of these panels. M, *Escherichia coli* 5S RNA was end-labelled with pCp(5'-32P). (**B**) The same Northern blots were stripped and reprobed with 20 base oligonucleotides complementary to the opal (left panel), amber (center panel) or ochre (right panel) mature tRNA transcripts under stringent conditions which minimize mismatches between the RNA/DNA hybrids by including a cold competitor oligonucleotide complementary to the wild type tRNA^{Trp} sequence. (**C**) The blots were hybridized with an oligonucleotide complementary to the intron of the pre-tRNA^{Ile} transcript as an internal control for loading. The band corresponding to end matured pre-tRNA^{Ile} is shown.

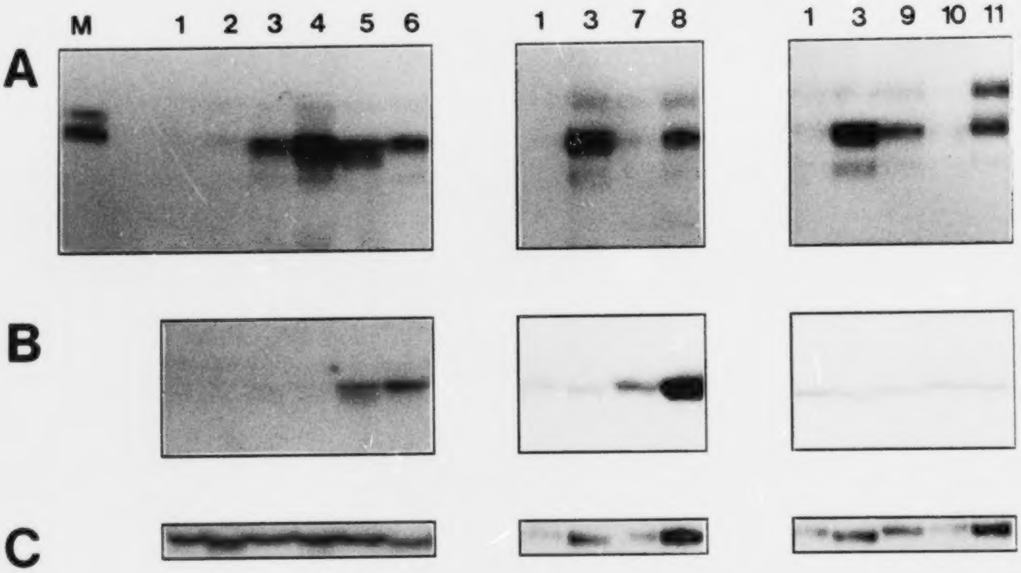

M
1
2
3
4
5
6
1
3
7
8
1
3
9
10
11
A
B
C

TABLE III-1

Suppressor phenotype of yeast strains when transformed with wild type and engineered $tRNA^{Trp}$ genes.

Putative suppressor alleles of a $tRNA^{Trp}$ gene	Suppressor phenotype		
	Amber	Ochre	Opal
op1	-	-	-*
op2	-	-	+
op2'	-	-	+
am1	++	-	-
am2	++	-	-
oc1	-	-	-
oc2	-	-	-
oc2'	-	-	-

The suppressor phenotypes of JG369-3B and JG113-5R transformed with the putative suppressor alleles of the $tRNA^{Trp}$ gene were determined as described in Atkin et al., 1990.

The plus (+) symbol indicates that suppression was observed only when strains were transformed with a high-copy number plasmid. The ++ symbol indicates that suppression was observed when strains were transformed with either a high-copy number plasmid or a single-copy plasmid. However, these high-copy number plasmids were toxic as judged by the slower rate of growth of cells transformed with these plasmids.

A minus (-) symbol indicates that suppression was not observed.

* A $tRNA^{Trp}$ gene with the same change in the anticodon as op1 was previously observed to suppress opal nonsense mutations by Kim et al. (1990) although no suppression was observed in our experiments.

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CHAPTER IV: Characterization of the tRNA^{Trp} genes of *Saccharomyces cerevisiae*

INTRODUCTION

Suppression, in *Saccharomyces cerevisiae*, has been studied for almost three decades. The isolation of nonsense suppressor mutations in yeast was first published by Hawthorne and Mortimer (1963). Since that time there have been extensive genetic screens to isolate additional nonsense suppressors in yeast. To date, all the nonsense suppressors in *S. cerevisiae* are alleles of genes encoding tRNA^{Tyr}, tRNA^{Ser}, tRNA^{Leu} or tRNA^{Gln} (reviewed in Sherman, 1982; Edelman and Culbertson, 1991). There are six other tRNA genes which should require only a single base change in the anticodon to create a tRNA capable of reading one of the nonsense codons. However, the isolation of spontaneous suppressors involving these tRNA genes has not been published. In one of these, the yeast tRNA^{Trp} gene, a single change in the CCA anticodon from C to U in either the first base or the second base of the anticodon should be sufficient to create an anticodon capable of reading either the opal or amber nonsense codons, respectively.

The cloning of a *S. cerevisiae* a gene for tRNA^{Trp} was originally reported by Kang et al. (1980). Attempts to construct an ochre suppressor *in vitro* have not yet been successful (Kim et al., 1990 and this thesis, Chapter III). An opal suppressor allele of this gene can be constructed by *in vitro* mutagenesis to change a single base in the anticodon. However suppression by the product of this gene is only seen when the gene is present on a multicopy plasmid (Kim et al., 1990). A gene with changes in both the anticodon and in the intron is a more efficient opal suppressor, but this gene must also be present on a multicopy plasmid to observe suppression (Atkin et al., 1990). It has also been demonstrated that an amber suppressor can be constructed by a single change in the CCA anticodon to CTA in this gene (Kim and Johnson, 1988; and this study). This gene was found to be an efficient suppressor when expressed from a single copy plasmid. These results suggest that we would not expect to be able to isolate ochre and opal suppressors of $tRNA^{Trp}$ by conventional genetic techniques. However, the ability to construct an efficient amber suppressor by a single engineered change suggests it may be possible to isolate amber $tRNA^{Trp}$ suppressors by conventional genetic techniques.

One species of $tRNA^{Trp}$ precursor accumulates in *rna1* mutants of *S. cerevisiae* (Ogden et al., 1984). This and the absence of reported yeast $tRNA^{Trp}$ suppressors in the literature, suggests there may be only one $tRNA^{Trp}$ gene. Alternatively, there may be several $tRNA^{Trp}$ genes and one is transcribed more efficiently than the others, or all copies are identical and required for $tRNA^{Trp}$ function. To examine these possibilities, the number of functional $tRNA^{Trp}$ genes was determined and the consequences of disrupting a functional copy of a $tRNA^{Trp}$ gene were examined.

MATERIALS AND METHODS

Materials:

Restriction enzymes, T4 DNA ligase, and *E. coli* DNA polymerase I, Klenow fragment were purchased from BRL. T7 DNA polymerase was purchased from Pharmacia, and Taq DNA polymerase was purchased from Perkin-Elmer/Cetus. Enzymes were used according to the manufacturer's instructions. Oligonucleotides were 5' end-labelled with [γ^{32}P]-ATP (4500 Ci/mmole), [α^{32}P]-dCTP (3000 Ci/mmole) was used in the preparation of oligo-labelled probes, and [α^{32}S]-dATP (500 Ci/mmole) was used for DNA sequencing. The oligonucleotides used for probing, sequencing and PCR were either purchased from the Regional DNA Synthesis Laboratory, University of Calgary, Canada, or synthesized in the Department of Microbiology DNA Synthesis Facility, University of Alberta.

Strains and genetic techniques:

Escherichia coli strain DH5α was used as a host for the propagation of plasmid DNA. M13 DNAs were propagated in *E. coli* strain JM105. DH5α and JM105 were propagated and transformed using standard methodology (Sambrook et al., 1989). The *S. cerevisiae* strains used in this study are listed in Table IV-1. Strains were constructed using standard yeast genetic techniques (Ausubel et al., 1989). Yeast transformations were performed using the lithium acetate protocol described in Ausubel et al. (1989).

DNA manipulation:

DNA was digested with restriction enzymes following the manufacturer's recommendations. Cloning, transformation, and extraction of plasmid DNA from *E. coli* was done using standard protocols (Sambrook et al., 1989). Yeast genomic DNA was prepared by the method of Ausubel et al. (1989). Isolation of intact yeast DNA, for pulse gel electrophoresis, was performed as described in Sambrook et al. (1989).

Preparation of and hybridization to Southern blots:

Yeast genomic DNA digested with the appropriate restriction enzyme was resolved on a 1.0% agarose gel. The DNA was transfered from the gel to Genescreen Plus following the manufacturer's (duPont Co.) instructions. Intact yeast chromosomes were resolved in a CHEF gel system. The gel was prepared for Southern transfer as follows. The gel was soaked in 0.25N HCl for 12 minutes; rinsed twice with distilled water; denatured for 60 minutes in 200-300 ml 0.5M NaOH, 1.5M NaCl; neutralized in 200-400 ml 0.5M Tris.HCl (pH 7.4), 1.5M NaCl for 60 minutes and then equilibrated in 6XSSC for 15 minutes. The subsequent transfer to Genescreen Plus was done according to the manufacturer's instructions. Southern blots were hybridized to end-labelled oligonucleotides using the hybridization conditions described in van Tol et al. (1988), with modifications (Wallace and Miyada, 1987).

The protocol of Klessig and Berry (1983) was used for Southern blot hybridization with oligolabelled probes.

Polymerase chain reaction and DNA sequencing:

Yeast genomic DNA, isolated from strain RJ293-13C, was used as the template for isolation of the yeast tRNA^{Trp} genes using primers A and B (Figure IV-3). Plasmid DNA with a 0.41 kilobase fragment of yeast DNA (containing a tRNA^{Trp} gene) was used to clone 5' and 3' flanking DNA sequences using primers C and D, and E and F (Figure IV-3), respectively. Extension was done with Taq DNA polymerase using the supplier's recommended conditions.

The tRNA^{Trp} genes were cloned into pUC119 and sequenced by the dideoxynucleotide chain termination method with T7 DNA polymerase, using the -20 universal primer and the reverse sequencing primer (Sambrook et al., 1989). The 0.41 kilobase fragment of yeast DNA in M13mp19 was sequenced by dideoxy-mediated sequencing reactions using the Klenow fragment of *E. coli* DNA polymerase I. The -20 universal sequencing primer and a custom primer complementary to 15 bases (12 base pairs upstream) of the tRNA^{Trp} were used for this purpose.

RESULTS

Determination of copy number and location of the tRNA^{Trp} genes in *S. cerevisiae.*

The copy number of tRNA^{Trp} genes in the haploid genome of *S. cerevisiae* was examined by genomic Southern analysis. Genomic DNA was isolated from four haploid yeast strains, RJ293-13C, JG369-3B, N123, JG113-5R, and aliquots were digested individually with *Bam*HI, *Eco*RI, *Hin*dIII, and *Pst*I. Figure IV-1 shows Southern blots of restriction digests of genomic DNA from RJ293-13C and JG369-3B. Southern transfers were first probed with an end-labelled oligonucleotide complementary to 21 bases of the 3' end of the gene (Figure IV-1A,C). Six bands of equal intensity were observed in each genomic digest, for each of the haploid yeast strains. Restriction fragment length polymorphisms (RFLP) were observed between each of the four strains analyzed herein. Figure IV-1A and C show the autoradiographs from Southern blots of genomic DNA from two of these strains (RJ293-13C and JG369-3B) digested by four different enzymes. A single RFLP can be observed between these two strains. The other two strains (data not shown) also exhibited polymorphisms.

To determine whether each of the six tRNA^{Trp} genes contain introns, Southern blots were probed with an end-labelled oligonucleotide which is complementary to ten bases 5' and ten bases 3' to the intron, or an oligonucleotide complementary to ten bases of intronic sequences and ten bases of exonic sequence surrounding the 3' splice junction. These hybridizations were done under conditions which will allow a maximum of one mismatch. A blot with mature $tRNA^{Trp}$ was included in the experiment, as a control. We have previously shown that the first end-labelled oligonucleotide hybridizes specifically to mature $tRNA^{Trp}$ and not intron-containing precursor (Atkin et al., 1990), while the second end-labelled oligonucleotide hybridizes only to intron-containing precursors under the experimental conditions used. In agreement with these previous experiments, the first end-labelled oligonucleotide hybridized to only the mature $tRNA^{Trp}$ (results not shown). The second end-labelled oligonucleotide hybridized to the same bands as the end-labelled oligonucleotide complementary to 21 bases of 3' end of the gene used to determine the copy number of the $tRNA^{Trp}$ gene. Thus, all of the $tRNA^{Trp}$ genes in *S. cerevisiae* appear to contain introns, and the 3' ends of the introns must be very similar.

The Southern blots were also probed with an end-labelled oligonucleotide complementary to 15 nucleotides 5' to the start of the mature coding sequences of the gene cloned by Kang et al. (1980). A single band was observed in each genomic digest for each strain (Figure IV-1B) suggesting that there is very little sequence homology in the 5' flanking sequences between the original member to be cloned (Kang et al., 1980) and the other members of this family of genes. No polymorphisms were seen in this case since the pattern of bands observed for RJ293-13C (Figure IV-1B), was identical to the patterns of bands observed for the Southern blots of genomic digests of DNA isolated from the three other strains.

The genomic locations of the tRNA^{Trp} genes were examined by probing Southern blots of intact yeast chromosomes separated by pulse field gel electrophoresis (Figure IV-2). The Southern blot of the yeast chromosomes was first probed with the end-labelled oligonucleotide complementary to the 3' end of a tRNA^{Trp} gene. Five bands were observed (Figure IV-2A), four of approximately equal intensity and one with roughly twice the intensity. The more intense band correlated to the expected positions of chromosomes XV and VII. Thus there may be two genes on one chromosome, or these genes may be located on separate chromosomes. The same Southern blot was hybridized with a 0.41 kb tRNA^{Trp} fragment sub-cloned from the 3.4 kb fragment cloned by Kang et al. (1980) to determine to which chromosome this gene maps. This probe hybridizes to chromosome XV or VII (Figure IV-2B). Thus, the first member of this gene family to be cloned maps to chromosome XV or VII, and the six tRNA^{Trp} genes in the haploid genome of *S. cerevisiae* are dispersed on five or six chromosomes.

These results are substantially in accordance with those already presented (Yesland et al., 1991). Similar experiments have been published, also showing Southern analysis of intact chromosomal DNA and restriction digests of genomic DNA from

RJ293-13C. Their analysis led the authors to suggest there are seven copies of the tRNA^{Trp} gene. This conclusion was based on the observation of five bands of approximately equal intensity and two fainter bands in the lane with the *Hin*dIII digest of the RJ293-13C genomic DNA probed with an end-labelled oligonucleotide. Figure 1A shows a *Hin*dIII digest of the RJ293-13C genomic DNA, also probed with an end-labelled oligonucleotide. Only six bands of equal intensity were observed. The fainter bands observed by Yesland et al., (1991) are most likely the result of incomplete restriction digests or a polymorphism within the strain. These authors also observed that there were no restriction length polymorphisms associated with this family of genes in the three yeast strains they examined. The work presented here supports the original estimate that the haploid genome of *S. cerevisiae* has a maximum of six copies of the tRNA^{Trp} gene (Kim and Johnson, 1988; and Atkin et al., 1990) and also demonstrates that restriction length polymorphisms can be associated with at least some of the members of this gene family.

Cloning of the tRNA^{Trp} genes

The sequence of one of the tRNA^{Trp} genes has been published (Kang et al., 1980). This sequence and additional 5' and 3' flanking sequences are shown in Figure IV-3. Based on this sequence, primers A and B were designed to clone each of the tRNA^{Trp} genes from the haploid yeast genome by the polymerase chain reaction (PCR). Yeast genomic DNA (isolated from RJ293-13C) was digested with *Hin*dIII and size fractionated on an agarose gel. DNA isolated from gel slices was used in different PCR reactions. Products were obtained only from the DNA of the expected size fractions. PCR products were not detected in any of the control reactions containing DNA from gel slices of size fractions which were not expected to contain tRNA^{Trp} genes. The products were cloned into pUC119 for sequencing in both directions. Multiple, independently isolated PCR products were sequenced for each size class. The sequences of the mature coding regions and the introns of the cloned tRNA^{Trp} genes were identical.

Replacement of a copy of a tRNA^{Trp} gene with *URA3*

To determine whether six copies of the tRNA^{Trp} gene are necessary for viability of haploid cells, a copy of this gene was replaced by the *URA3* gene. The first member of the tRNA^{Trp} gene family to be cloned was chosen for gene replacement. No restriction length polymorphisms are associated with this tRNA^{Trp} gene (see above). This raises the possibility that this gene is tightly flanked by essential sequences. To ensure that only the tRNA^{Trp} gene was replaced by the *URA3* gene, PCR was used to clone the 5' and 3' flanking DNA immediately adjacent to this tRNA^{Trp} gene and create restriction sites for insertion of the *URA3* gene between these flanking sequences. Primers C and D (Figure IV-3) were used to clone a 155 base pair fragment from the 5' flank. These primers were designed to create *Hin*dIII and *Eco*RI restriction sites. The 149 base pair fragment from the 3' flank was cloned using primers E and F (Figure IV-3). These primers were designed to create *Pst*I and *Kpn*I restriction sites. A 0.9 kilobase *Sma*I-*Pst*I fragment (with an *Eco*RI linker added to the *Sma*I end) capable of complementing the *ura3-52* mutation was cloned between the 5' and 3' tRNA^{Trp} flanking sequences. The resultant *Hin*dIII-*Kpn*I fragment, with the *URA3* gene cloned between the 5' and 3' tRNA^{Trp} flanking sequences, was used to transform a diploid strain, ALA11, constructed for this study (see materials and methods).

The single-step gene replacement of a chromosomal copy of the tRNA^{Trp} gene with the 1.2 kilobase *Hin*dIII-*Kpn*I fragment was performed essentially as described by Rothstein (1983). The diploid recipient, ALA11, was used in case the disruption was a lethal event in a haploid strain. Transformants that were prototrophic for uracil were selected and examined for a tRNA^{Trp}::*Ura3* disruption by Southern analysis. Genomic DNA from untransformed and transformed diploids was digested with *Bam*HI and hybridized with a *URA3*-specific probe. The same blots were stripped and hybridized with the 0.41 kilobase tRNA^{Trp} probe. DNA from the parental diploid (ALA11) produced a 15 kilobase *Bam*HI band when hybridized to the oligolabelled *URA3* probe. DNA from the uracil-prototrophic transformants produced two bands of homology to the *URA3* probe, a 15.0 kilobase band and a 6.0 kilobase band (Figure IV-4A). A band in the same position as the faster migrating band also hybridizes to the 0.41 kilobase tRNA^{Trp} probe (data not shown) indicating this band represents the targeted replacement event.

The parental diploid (ALA11) and the disrupted diploids (ALA12, ALA13, and ALA14) were sporulated, and the asci were dissected. Tetrad analysis showed that in all 32 asci which produced four viable spores, URA segregated 2+:2- (Figure IV-4B). This demonstrates that six copies of the tRNA^{Trp} genes in the haploid yeast genome are not essential for growth, since the URA+ spores were viable, but had only five copies of the tRNA^{Trp} gene. Thus, a maximum of five copies of this gene are sufficient for viability.

DISCUSSION

Herein, an attempt was made to determine why suppressor alleles of a *S. cerevisiae* tRNA^{Trp} gene isolated by conventional genetic techniques have not been reported. Nonsense suppressor alleles of tRNA^{Tyr}, tRNA^{Ser}, tRNA^{Leu} and tRNA^{Gln} have been isolated by traditional methods (reviewed in Sherman, 1982; Edelman and Culbertson, 1991). A single copy of a gene for any of these tRNAs, mutated such that the anticodon can recognise nonsense alleles, is sufficient for suppression. A single change in the CCA anticodon of a gene for tRNA^{Trp} to CTA should be sufficient to create a tRNA capable of interacting with amber nonsense codons. A gene with this change has been constructed and was found to be an efficient suppressor when expressed from a single copy plasmid (Kim and Johnson, 1988; and this study, Chapter III). Yeast cells which have a single copy of this gene have growth rates similar to yeast cells transformed with a single copy of the wild type gene, although this amber suppressor is toxic when present on a multicopy plasmid (Kim et al., 1990 and Atkin et al., 1990). The absence of amber suppressor alleles of this gene isolated by conventional genetic techniques in the literature suggested that there may only be a few copies of the tRNA^{Trp} gene in the yeast genome and that all may be essential. In this paper this possibility has been addressed by identification and partial characterization of all of the tRNA^{Trp} genes in yeast. Herein, data are presented which show there are six copies of this gene in the haploid genome of *S. cerevisiae*. All copies were cloned and shown to contain identical introns. The possibility that all six copies are required was tested by replacing a copy with the *URA3* gene. The results of this experiment clearly show that a maximum of five tRNA^{Trp} genes are sufficient for viability of haploid yeast cells.

The ability to construct functional amber tRNA^{Trp} suppressors *in vitro* and the requirement for a maximum of only five of the six tRNA^{Trp} genes for viability of haploid yeast cells suggest it should be possible to isolate tRNA^{Trp} amber suppressors by conventional genetic techniques. A direct test of this suggestion would be to replace a copy of a tRNA^{Trp} gene with an *in vitro* constructed amber suppressor allele. This was tried with the am1 gene (unpublished results). Construction of this gene was described in Chapter III. Putative transformants of ALA11 were selected first for suppression of the *trp1-1* amber allele, and then for suppression of *met8-1*. No transformants with this gene were recovered. This suggests two possibilities. Gene replacements may have occurred, but not enough suppressor was produced to be able to select the suppressor, possibly because the genes are not active enough in their chromosomal context to produce suppression. Alternatively, the *Eco*RI linkers used to clone this gene provided enough heterology at the ends of the fragments to prevent site-specific recombination. These possibilities can be tested by repeating the experiments with a fragment containing an amber suppressor tRNA^{Trp} gene with native ends and a reporter gene in the 5' or 3' flanking sequence.

The absolute sequence identity observed for the introns of the yeast tRNA^{Trp} genes is unusual. Polymorphisms have been observed in the sequences of the introns encoded by members of other tRNA gene families in yeast. There appears to be little selective pressure on the sequence of introns in tRNA genes. Profound changes can be made to the sequence of the introns in tRNA genes without affecting their removal from pre-tRNA. Only mutations which affect the secondary structure of the precursor, or prevent the formation of the single stranded loop at the 3' splice junction have an effect on intron removal (reviewed in Atkin et al., 1990). Thus the absolute sequence identity observed amongst the introns of the tRNA^{Trp} genes, despite the limited selective pressure and the dispersed locations of these genes, implies that ectopic conversion likely occurs between the members of this gene family.

Ectopic conversion has been shown to occur between three genes encoding two serine tRNA isoacceptors located on different chromosomes or chromosome arms in *Schizosaccharomyces pombe* (Kohli et al., 1984; and Amstutz et al., 1985). All three of these genes are closely associated with methionine initiator tRNA genes and give rise to dimeric primary transcripts. Conversion events between these genes were found to be limited to the sequences within the dimeric tRNA genes. Changes were never seen within the flanking DNA. In addition, the transfer of information was undirected. Ectopic conversion is also thought to occur between the $tRNA_4^{Ser}$ and $tRNA_7^{Ser}$ genes of *Drosophila melanogaster* (Cribbs et al., 1987). The sequences of these genes are 96% homologous, differing only at the first position of the anticodon and two other sites. This unusual degree of homology suggests these genes are undergoing concerted evolution.

The homology observed for the family of $tRNA^{Trp}$ genes in *S. cerevisiae* and their dispersed locations suggest that they could be an excellent model system for studying ectopic conversion events between members of a family of genes in their native chromosomal context. Key to these studies will be the development of a sensitive, quantitative assay for suppression by amber alleles of the $tRNA^{Trp}$ genes. With such an assay system it would be possible to monitor conversion at $tRNA^{Trp}$ loci which have been replaced with amber suppressor alleles.

TABLE IV-1

Yeast strains used in these studies.

Strain	Genotype	Source
RJ293-13C	*MAT**a**, his4-260, leu2-3,112, ura3-52*	J. Johnson
JG369-3B	*MATα, ade2-1, lys2-1, can1-100, met8-1, trp1-1, leu2-2, his4-260, ura3-52*	J. P. Gelugne
JG113-5R	*MATα, ade2-1, can1-100, met8-1, trp1-1, leu2-2, his4-260*	J. P. Gelugne
N-123	*MAT**a**, his1 (rho+, ome-,* CHL^S, ERY^S, OLI^S)	R. C. von Borstel
ALA1-12B	*MATα, lys2-1, met8-1, trp1-1, his4-260, ura3-52*	this study
ALA2-28C	*MAT**a**, ade2-1, met8-1, trp1-1, ura3-52, ilv1*	this study
ALA11	ALA1-12B x ALA2-28C	this study

FIGURE IV-1

Southern blot analysis of genomic DNA from *Saccharomyces cerevisiae* strain RJ293-13C and JG369-3B.

A. The Southern blot of genomic DNA from RJ293-13C digested with *Bam*HI (B), *Eco*RI (E), *Hin*dIII (H), and *Pst*I (P) was hybridized with an end-labelled oligonucleotide probe which is complementary to 21 bases of the antisense strand at the 3' end of the gene.

B. The same Southern blot was hybridized to a probe which is complementary to 15 bases, 12 nucleotides upstream of the 5' mature coding sequences of the $tRNA^{Trp}$ gene cloned by Kang et al., (1980).

C. An autoradiograph of a Southern blot of genomic DNA from JG369-3B digested with *Bam*HI, *Eco*RI, *Hin*dIII, and *Pst*I hybridized with the same probe as in A.

The positions of the λ *Hin*dIII size markers have been indicated.

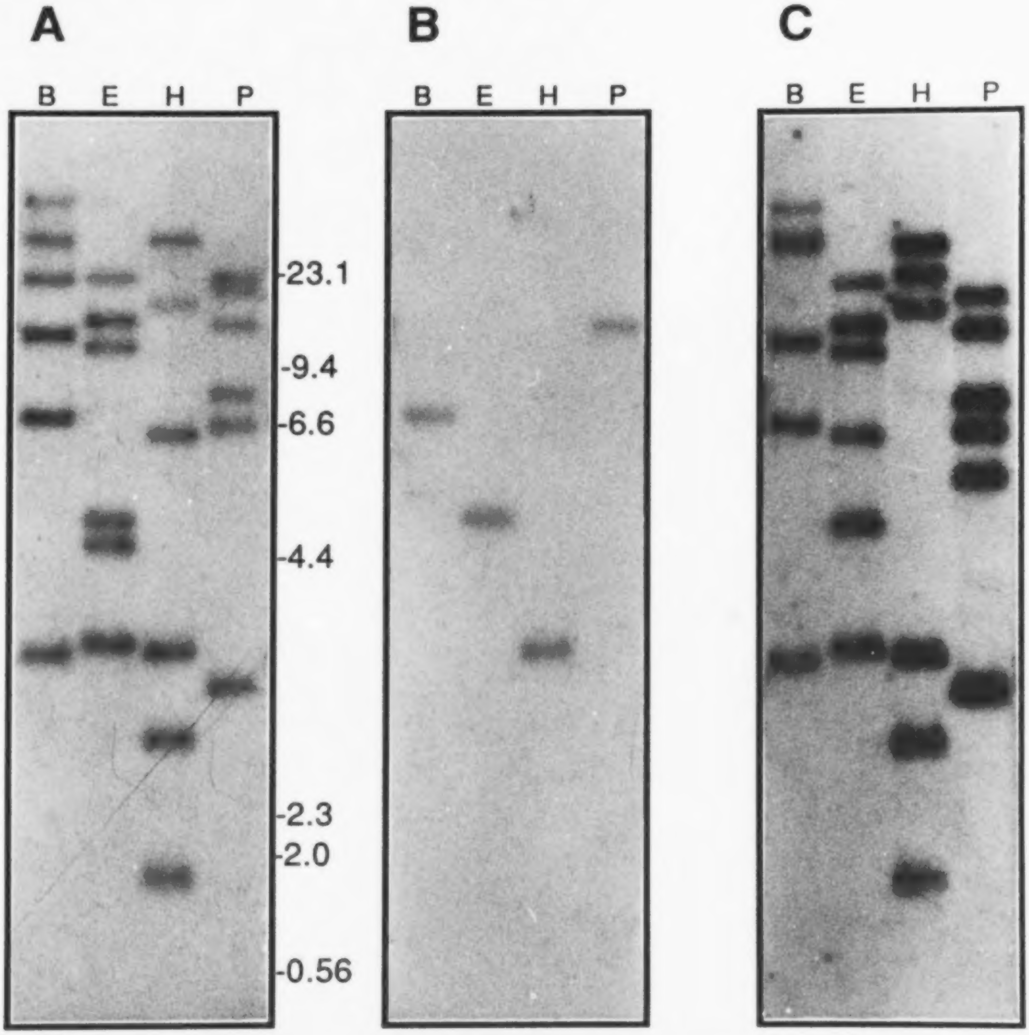

A
B
C
B E H P
B E H P
B E H P
-23.1
-9.4
-6.6
-4.4
-2.3
-2.0
-0.56

FIGURE IV-2

Southern blot hybridization of yeast chromosomes separated by pulse gel electrophoresis.

A. The Southern blot was hybridized with an end-labelled oligonucleotide complementary to 21 bases of the antisense strand in the 3' end of the $tRNA^{Trp}$ gene.

B. The same Southern blot was hybridized to an oligo-labelled 0.41kb fragment of yeast DNA containing a $tRNA^{Trp}$ gene.

The location of the yeast chromosomes is indicated. The position that the individual yeast chromosomes migrated to was determined from the ethidium bromide stained, agarose gel before the Southern transfer.

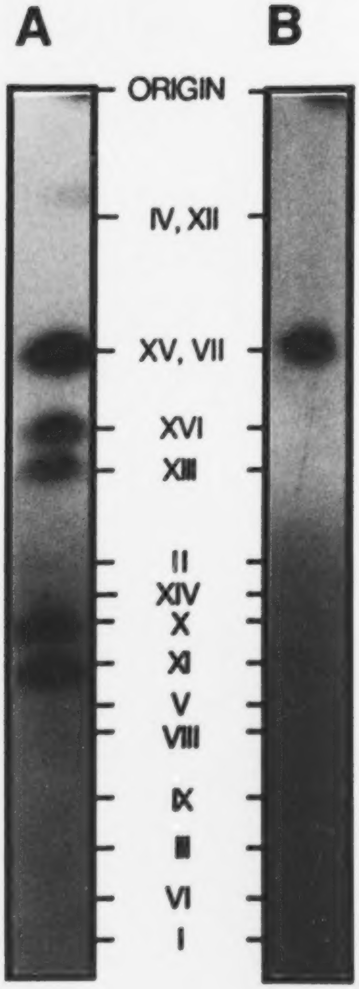

A
B
ORIGIN
IV, XII
XV, VII
XVI
XIII
II
XIV
X
XI
V
VIII
IX
III
VI
I

FIGURE IV-3

Sequence of a cloned tRNA^{Trp} gene (modified from Kang et al., 1980). The location of the tRNA^{Trp} gene is indicated by the stipled line below the sequence. Primers A, B, C, D, E and F were used for PCR. Primers A and B were used to clone all copies of the tRNA^{Trp} gene. Primers C and D were used to clone a 155 base pair fragment from the 5' flank of the tRNA^{Trp} gene. A 149 base pair fragment was cloned from the 3' flank of the tRNA^{Trp} gene using primers E and F.

```
              HindIII
     C 5'CCCT AAGCTTGGTG AACTATATTC 3'
5' GAATTCCCCT GTTCTTGGTG AACTATATTC TAGTTACTAA TTATCAAAAC TGACGATAAC TAGGAGCCGT TTTAAAGATA CAGCTTTCGG

                                                                                 EcoRI
                                                              D 3' G AGTTACCATC TCTTAAGCTG A 5'
                                                   A 5' G AAGCGGTGGC TCAATGGTAG AG 3'
   GGAGACAAGT AAGAATTTAA TTCTTGGATA TTTCAAGATG AAGCGGTGGC TCAATGGTAG AGCTTTCGAC TCCAATTAAA TCTTGGAAAT
                                                        \\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\

                                  PstI
                         E 5' GGGCT GCAGGTTCAA TTCCTGT 3'
                               B 3' CGTCCAAGTT AAGGACAGGC AAAGT 5'
   TCCACGGAAT AAGATTGCAA TCGAAGGGTT GCAGGTTCAA TTCCTGTCCG TTTCATTTTT TATACTGCAA GTGATTAATT TAAGGATGGT
   \\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\

                                                                                 KpnI
                                                           F 3' GCTA ACGAAAAATA CCATGGAAAA 5'
   TAAATGAAGG AAACGGTGTA CTAAATGTGC AGGGGCGTCT CTGATGTCGC GTTTACCGAT TGCTTTTTAT TGTACCTTTT CTATTCTTTT


   TTTTTTCGGT TCAATTCTTG TAAGTCCACT GACGGACAAG AAATGTTGGA ATTC 3'
```

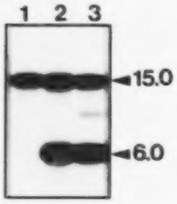
FIGURE IV-4

Single-step gene replacement of a chromosomal copy of a tRNA^{Trp} gene with the *URA3* gene.

A. An autoradiograph of a Southern of genomic DNA of the parental diploid, ALA11 (lane 1); and two uracil-prototrophic transformants, ALA12 (lane 2) and ALA 13 (lane 3) restricted with *Bam*HI and hybridized with an oligolabelled *URA3* probe. The sizes of the bands observed were determined by comparison to a λ *Hin*dIII size marker.

B. Tetrad analysis of the parental diploid, ALA11 and three uracil-prototrophic transformants ALA12, ALA13, and ALA14.

A



B

YEAST STRAIN	NUMBER OF TETRADS	SEGREGATION PATTERNS OBSERVED FOR		
		ADE+/ade-	URA+/ura-	
ALA11	18	2+/2-	0+/4-	In all tetrads
ALA12	9	2+/2-	2+/2-	"
ALA13	16	2+/2-	2+/2-	"
ALA14	7	2+/2-	2+/2-	"

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CHAPTER V: SUMMARY AND CONCLUSIONS

The original goal of this thesis was to examine nonsense suppression in lines of *Drosophila melanogaster* which had been transformed with an opal suppressor allele of a *Saccharomyces cerevisiae* $tRNA^{Trp}$ gene. A molecular approach to the study of suppression in Drosophila was taken since it has not been possible to isolate nonsense suppressor alleles of tRNA genes by conventional genetic techniques.

P-element mediated transformation has been used to introduce the *Sup-3e* gene of *Schizosaccharomyces pombe* into Drosophila (Molnar et al., 1988). *Sup-3e* is an opal suppressor allele of a *S. pombe* $tRNA^{Ser}$ gene which has been shown by an immunological assay to suppress an opal mutation in the *adh* gene of Drosophila *in vitro* (Kubli et al., 1982). No suppression was observed *in vivo*, however (Molnar et al., 1988). The lack of observed suppression was most likely due to one of two reasons. First, the heterologous $tRNA^{Ser}$ gene of *S. pombe* was not transcribed, processed, aminoacylated, or could not interact efficiently with the Drosophila translational machinery. Alternatively, the heterologous $tRNA^{Ser}$ gene of *S. pombe* was active in Drosophila, but serine was an inappropriate amino acid replacement since the opal mutation in the *adh* gene was a single base change in a tryptophan codon. Two approaches were taken to distinguish between these two possibilities. First, opal suppressor alleles were constructed by *in vitro* mutagenesis of a *Drosophila melanogaster* $tRNA^{Ser}$ gene and cloned into Drosophila by P-element mediated transformation (Pappu, 1990). Secondly, an analogous approach was taken to transform Drosophila with opal suppressor alleles of a $tRNA^{Trp}$ gene. Since the $tRNA^{Trp}$ gene of Drosophila had not been cloned, a $tRNA^{Trp}$ gene of *S. cerevisiae* was used. Opal alleles of this gene were constructed (Atkin et al., 1990) and sub-cloned into Carnegie 20, a P-element vector. These constructs have been transformed into Drosophila (data not shown), and await genetic and molecular analysis.

During the preparation of the opal suppressor $tRNA^{Trp}$ genes for transformation into Drosophila, observations were made in yeast which have implications for the importance of the secondary structure of pre-tRNA in intron removal *in vivo*. These observations changed the focus of this thesis project.

Putative opal suppressor $tRNA^{Trp}$ genes were constructed using site-directed oligonucleotide *in vitro* mutagenesis. No suppression of opal nonsense mutations was observed in yeast strains transformed with the gene that has the single base change in the anticodon sequence; however suppression was observed in yeast strains transformed with the gene that has both the anticodon and the intron changes. It was subsequently observed that intron-containing precursors tend to accumulate in a yeast strain transformed with suppressor constructs having only the requisite anticodon change. Thus the difference in the suppressor phenotype, of cells transformed with the putative opal suppressor tRNA^{Trp} genes, appears to be due to less effective processing of the intron from precursors from the gene containing only the requisite anticodon change. It was hypothesized that this may reflect an altered secondary structure of the precursor in which base-pairing at the 3' splice junction inhibits cleavage by the tRNA endonuclease. The hypothesis was tested by introducing additional changes into the anticodon and the intron of the *S. cerevisiae* tRNA^{Trp} gene, which were expected to either inhibit or encourage base-pairing at the 3' splice site. Mutations in the anticodon of the tRNA^{Trp} gene, which should allow base pairing at the 3' splice site, were shown to affect processing of a pre-tRNA^{Trp} at the intron removal step. Second site mutations in the intron, designed to inhibit base pairing at the 3' splice junction, could restore recognition and cleavage by the tRNA splicing endonuclease if another step in the processing pathway has not been disrupted.

As mentioned in the preceding paragraph, we did not observe suppression in yeast cells transformed with putative opal suppressors which have only the requisite anticodon change. However, Kim et al. (1990) have observed suppression with a similar construct. This difference may be due to one of several reasons. First, although the same gene was used in both studies it was sub-cloned on different size fragments. A 0.41 kb fragment was used in the experiments presented herein and the others used a 0.9 kb fragment. Since the flanking DNA of tRNA genes has been shown to affect their expression (reviewed in Chapter 1), an enhancer sequence may have been deleted from the flank of the $tRNA^{Trp}$ gene on the 0.41 kb fragment, or the gene may have been cloned next to plasmid sequences which inhibit the expression of this gene. Secondly, different plasmids were used. The 0.41 kb fragment of yeast DNA with the op1 gene was cloned into YRp17. The larger fragment was cloned into YCp19 from which a 1.6 kb *Xho*I fragment (which includes the *CEN4* sequence) had been deleted. Third, different methods for transformation of yeast were used. The yeast strains used in this study were transformed with the polyethylene-glycol-induced method, while the lithium acetate mediated method of transformation was used by Kim et al. (1990). The method of transformation does seem to affect the expression of $tRNA^{Trp}$ suppressor genes. Strains of yeast transformed by the first method must be selected first for presence of the plasmid before suppression can be detected (results not shown). However, suppressors can be selected directly after transformation by the second method (Kim and Johnson, 1988). Thus, the reason for the difference observed in the ability of yeast cells transformed with a $tRNA^{Trp}$ gene having a UCA anticodon sequence to suppress is not clear, but it may be due to one or a combination of the reasons discussed above.

The wild type $tRNA^{Trp}$ gene also can suppress opal nonsense mutations, but not amber or ochre nonsense mutations. This suppression is very weak. Growth on plates selecting for opal nonsense suppression was observed only after extended incubation. In addition, suppression by the wild type $tRNA^{Trp}$ was dependent upon the genetic background. Suppression was seen in *S. cerevisiae* strains JG369-3B and RJ239-13C, but not in strain JG113-5R. The difference in genetic backgrounds may result in either inefficient modification or a reduced translational fidelity. Modified nucleotides at the wobble position can affect codon choice (Bjork et al., 1987). The 3' base of the anticodon of $tRNA^{Trp}$ is normally a 2'-O-methylcytidine (Keith et al., 1971). This modification could be important for restricting the codon choice of $tRNA^{Trp}$ to the UGG tryptophan codon. It may be possible to take advantage of the different genetic backgrounds to clone the gene(s) involved in allowing wild type $tRNA^{Trp}$ to read opal nonsense codons.

The $tRNA^{Trp}$ is not the first wild type tRNA which has been observed to suppress nonsense mutations in *S. cerevisiae*. Normal *S. cerevisiae* glutamine $tRNA_{UUG}$ and glutamine $tRNA_{CUG}$ can suppress ochre and amber nonsense mutations, respectively, when the genes for these tRNAs are present on a multicopy plasmid (Pure et al., 1985; and Weiss and Friedberg, 1986). This naturally-occurring suppression in normal cells has been attributed to unconventional wobble base pairing between a base in the anticodon and the first base in the codon rather than the third (Pure et al., 1985; Weiss and Friedberg, 1986; Edelman and Culbertson, 1991). In most eukaryotes, CAA and CAG code for glutamine, however in certain ciliated protozoa, UAA and UAG code for glutamine. These organisms have evolved glutamine tRNAs with UUA and CUA anticodons capable of reading the UAA and UAG codons, respectively, using standard anticodon:codon interactions. The ability of the normal glutamine tRNAs to decode these codons has led to the suggestion that these tRNAs may be the evolutionary progenitors for the glutamine tRNAs which decode the UAA and UGA codons in these protozoa (Weiss and Friedberg, 1986). In mitochondria, UGA is used to code for tryptophan (Sprinzl et al., 1987), and for this reason the ability of yeast cytoplasmic $tRNA^{Trp}$ to decode UGA codons weakly may also be evolutionally significant. The progenitors of mitochondria may have possessed $tRNA^{Trp}$ with a CCA anticodon, which decodes the universal tryptophan codon UGG. Analogous to the scenario suggested for the glutamine tRNAs of protozoa, the UGA stop codon entered the coding region of essential genes by random genetic drift in the progenitors of the present day mitochondria. In these progenitors, $tRNA^{Trp}$ would be able to decode weakly the UGA stop codon. Eventually, there would be selection for altered $tRNA^{Trp}$ with enhanced stability of codon-anticodon pairing, and this would select against the UGA codon as a translational terminator. Ultimately, mutation of a C to a U in the first position of the anticodon would generate a $tRNA^{Trp}$ capable of decoding UGA at a high efficiency. An alternate, and perhaps more attractive explanation, may be that UGG and UGA both coded for tryptophan in some ancient genetic code. The cells which became the progenitors of the modern day mitochondria evolved to use UGA to code for tryptophan, while the cells which became the progenitors of the eukaryotic nuclei lost the ability to recognize UGA as a codon for tryptophan. The ability of wild type cytoplasmic $tRNA^{Trp}$ to decode, weakly, the UGA codon may be a remnant of this ancient genetic code. It should be possible to test these hypotheses by making second site changes in a gene for cytoplasmic $tRNA^{Trp}$ based on the sequence of the mitochondrial $tRNA^{Trp}$ gene, and then assaying suppressor efficiency to determine whether these changes affect the ability of the $tRNA^{Trp}$ to decode the UGA codon and vise versa.

In addition to the opal suppressor alleles of $tRNA^{Trp}$, putative amber and ochre suppressor alleles were constructed. Kim and Johnson (1988) first showed that it is possible to construct amber suppressor alleles of a $tRNA^{Trp}$ gene by site-directed *in vitro* mutagenesis. They demonstrated that a $tRNA^{Trp}$ gene with the appropriate anticodon change encoded a specific suppressor of amber nonsense mutations. In addition, the gene only had to be present on a yeast centromeric (ie. single copy) plasmid to observe suppression. Their results have been confirmed herein and, in addition, it has been shown that the amber suppressor is toxic to yeast when it is present on a multicopy plasmid. A gene with the required anticodon change and a second change in the five base complementary intron sequence, which restores conventional base pairing potential for the transcripts, also encoded an active amber suppressor. Functional ochre suppressor alleles of a *S. cerevisiae*

$tRNA^{Trp}$ gene have not yet been constructed. Cells transformed with the gene that has only the requisite anticodon change do not contain detectable levels of mature suppressor tRNA. *In vitro* splicing assays suggest that the precursor from this gene is inefficiently recognized and cleaved by the tRNA splicing endonuclease. Subsequent second site changes in the intron of this gene, which should allow the precursor from this gene to fold into a secondary structure in which the 3' junction is located in a single stranded region, did not result in alleles encoding active ochre suppressors. In the first case, second site changes in the five base pair sequence of the intron complementary to five base pairs in the anticodon region resulted in a precursor which was end-processed aberrantly. The precursor of a second gene containing a different base change in the intron (oc2') was recognised and cleaved by the tRNA splicing endonuclease *in vitro*, but was not processed *in vivo*. These results suggest it may be possible to construct an ochre suppressor allele of a *S. cerevisiae* $tRNA^{Trp}$ gene by deleting the intron in a gene with the requisite anticodon change.

Although it was demonstrated that alleles of a $tRNA^{Trp}$ gene encoding functional opal and amber suppressors can be constructed, it is still not known which amino acid these suppressors insert in response to opal and amber nonsense codons, respectively. In *Escherichia coli*, amber, ochre and opal $tRNA^{Trp}$ suppressors insert glutamine instead of tryptophan in response to the appropriate nonsense codon (Soll, 1974; and Yaniv et al. 1974). Thus a change in the anticodon of the *E. coli* tRNA^{Trp} alters the identity of this tRNA. The same may be true for yeast tRNA^{Trp}. Further experiments will be necessary to determine which amino acid the yeast opal and amber tRNA^{Trp} suppressors are charged with. The amino acid that tRNA (transcribed from tRNA^{Tyr}, tRNA^{Ser} and tRNA^{Leu} suppressor genes) is acylated with has been determined indirectly by determining the amino acid inserted at nonsense codons in iso-1-cytochrome c from yeast (reviewed in Sherman et al., 1982). This approach could be used to determine the amino acid that suppressor tRNA^{Trp} is charged with.

Southern analysis of yeast genomic DNA was used to show that there are six copies of the tRNA^{Trp} gene and that each gene encodes an intron. These genes were found to map onto 5 or 6 of the yeast chromosomes. Analysis of the tetrads from a diploid in which a copy of the tRNA^{Trp} gene had been replaced by *URA3* demonstrated that a maximum of five copies of this gene are sufficient for viability of haploid yeast cells. This raises the question; what is the minimum number of copies of the tRNA^{Trp} gene required for viability of haploid yeast cells? This question could be answered by systematically replacing the other copies of the tRNA^{Trp} gene with selectable yeast markers and analysing the tetrads from the resultant diploid parent. In order to take this approach it will be necessary to clone the flanking DNA for each of the genes.

Attempts were also made to replace a tRNA^{Trp} gene with an amber suppressor allele of this gene. These attempts were unsuccessful. Either the gene replacement did not occur, or it did occur but the level of expression of this gene was not high enough to detect amber suppression. Further experimentation will be necessary to distinguish between these potential explanations. However, these observations raise the possibility that suppressor alleles of the $tRNA^{Trp}$ gene, obtained by conventional genetic techniques, have not been reported because the level of gene expression from the genes in their native chromosomal locations is not high enough to detect amber suppression. If this is true, amber suppression by a suppressor allele of $tRNA^{Trp}$ in its native chromosomal location may be observable in the presence of an allosuppressor such as *upf1* in yeast (Leeds et al., 1991).

Cloning of the flanking DNA will also allow *in vitro* and *in vivo* analysis of whether expression of the individual $tRNA^{Trp}$ genes is modulated by their flanking sequences. In *S. cerevisiae*, the genes for $tRNA^{Leu}$ contain a pentanucleotide sequence in their 5'-flank which has been demonstrated to enhance the expression of these genes (Raymond et al., 1985). A similar sequence precedes many of the *S. cerevisiae* tRNA genes. However, whether this sequence modulates the transcription of all the tRNA genes which it precedes is not known yet. This sequence has not been found in the 5' flank of the $tRNA^{Trp}$ gene originally cloned by Kang et al. (1980). It is possible that expression of the $tRNA^{Trp}$ genes is modified by an as of yet uncharacterized sequence. Comparison and deletion analysis of the flanking sequences will reveal if the expression of these genes is influenced by the flanking DNA.

PCR was used to clone each of the tRNA^{Trp} genes. The genes were all found to encode a 34 base pair intron. The sequence of the exons and the intron of each gene was found to be identical. The homology observed between the introns is unique amongst the intron-containing families of tRNA genes (reviewed in Chapter I). Since there appears to be little selective pressure on the sequence of introns in tRNA genes (reviewed in Chapter I) and the tRNA^{Trp} genes are dispersed, these genes may be undergoing ectopic conversion to maintain the sequence identity.

This family of genes may be a good model system for the study of ectopic recombination in *S. cerevisiae*. Most studies on ectopic recombination in yeast involve examination of events between a gene at its natural position and a copy of the gene which has been inserted into a different locus by gene replacement (reviewed in Petes and Hill, 1988). Although these studies have provided a lot of valuable information, it is still necessary to show that these results are representative of the recombination events that occur between genes at their normal loci. There is some evidence that the rate of conversion is lower between genes at their native chromosomal loci than expected from studies involving artificial duplications. Meiotic rates of conversion between Ty elements at different chromosomal locations in diploid yeast strains is considerably less than the rates detected in studies using artificial duplications (Petes and Hill, 1988). The $tRNA^{Trp}$ family of genes may be another excellent candidate for a model system for such studies because of the homology observed between the members of this family and their dispersed loci in the yeast genome. This would depend on the development of an assay sensitive enough to monitor suppression by an amber suppressor allele of a $tRNA^{Trp}$ gene at its native chromosomal locus. Once such an assay had been developed, a similar approach to the one used to assay recombination between the $tRNA^{Ser}$ genes of *S. pombe* could be employed.

The first direct evidence of heterochromosomal gene conversion came from studies with the *S. pombe* serine tRNA genes (Kohli et al., 1984; and Amstutz et al., 1985). *Sup9+* revertants in a diploid homozygous for a *sup9-UGA* mutation were often found to contain sequences from the non-allelic genes *sup3+* or *sup12+*. Two genetic screens were set up to explore these observations further. The first allowed direct selection for intergenic convertants. Active suppressors were selected in a strain homozygous for an inactive opal suppressor allele of *sup3*. The second screen selected for loss of suppressor activity. These experiments demonstrated that information transfer occurred between all possible pairs of the three genes and in both directions.

The approach used in this thesis to construct the amber and opal suppressor alleles of a $tRNA^{Trp}$ gene can be applied to other genes for which suppressor alleles have not yet been isolated. The ultimate goal of this would be to construct a complete set of suppressors to be used as a tool in studies of protein structure and function. In *S. cerevisiae* amber suppressor alleles of the $tRNA^{Tyr}$, $tRNA^{Ser}$, $tRNA^{Leu}$, $tRNA^{Gln}$, $tRNA^{Phe}$, and $tRNA^{Trp}$ genes have either been isolated by traditional techniques, or constructed *in vitro*. Ochre suppressor alleles of $tRNA^{Tyr}$, $tRNA^{Ser}$, $tRNA^{Leu}$, $tRNA^{Gln}$ and $tRNA^{Phe}$, and opal suppressor alleles of $tRNA^{Leu}$, $tRNA^{Ser}$, and $tRNA^{Trp}$ are available (Sherman, 1982; Edelman and Culbertson, 1991; Masson et al., 1987; Kim and Johnson, 1988; Atkin et al., 1990; and Kim et al., 1990). This is a good start for the construction of a set of suppressors which could insert one of each of the twenty amino acids in response to a nonsense codon. Such a set of suppressors has been constructed from *E. coli* tRNA genes (Normanly et al., 1986; Kleina et al., 1990; and Normanly et al., 1990). These suppressors were constructed to provide a direct method for examining the effects of many single amino acid substitutions at specified positions in proteins.

Construction of a set of yeast tRNA suppressors which could insert each of the twenty amino acids in response to a nonsense codon would involve the following steps. First, clones would have to be obtained of a tRNA gene for each of the amino acids. Second, oligonucleotide directed *in vitro* mutagenesis would provide a direct approach to making the appropriate anticodon change(s).

Third, the modified genes would have to be tested for their ability to suppress existing nonsense mutations and insert the correct amino acid. In *E. coli*, the specificities of the suppressor tRNAs were determined by N-terminal sequence analysis of dihydrofolate reductase to determine which amino acid had been inserted by the suppressor tRNAs at the position of an amber codon (Normanly et al., 1990). An analogous system could be developed for yeast. Finally, genes which suppress inefficiently or insert an incorrect amino acid would have to be modified to increase efficiency or to interact with the correct aminoacyl synthetase.

The research presented in this thesis has raised questions about the involvement of $tRNA^{Trp}$ in the evolution of the use of the UGA codon to specify tryptophan in the mitochondria, the potential of constructing functional ochre $tRNA^{Trp}$ suppressors, the aminoacylation of the amber and opal suppressor tRNAs, and the control of $tRNA^{Trp}$ gene expression. In addition, it may be feasible to use this gene family for studies of ectopic recombination, and to construct a set of eukaryotic suppressors to be used in studies of protein structure and function.

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